UNIVERSIDADE FEDERAL DO PARANÁ

LEANDRO FREIRE DOS SANTOS

THE PHARMACOLOGICAL POTENTIAL OF THE MACROMYCETES (*CORDYCEPS SINENSIS* AND *PLEUROTUS OSTREATUS*) CULTIVED BY SUBMERGED FERMENTATION

CURITIBA
2013

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Tese apresentada como requisito parcial à obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia/Processos Biotecnológicos, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, área de concentração: Saúde Humana e Animal, Setor de Tecnologia, Universidade Federal do Paraná.

Orientador: Dr. Carlos Ricardo Soccol
Co-orientadora: Dr^{a}. Rosália Rubel

CURITIBA
2013

TERMO DE APROVAÇÃO

LEANDRO FREIRE DOS SANTOS

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Dedicado este trabalho ao meu Pai, Claúdio, minha mãe, Branca, meu irmão Tiago, minha cunhada Mônica e a minha amada esposa Priscila por estarem sempre presentes. Amor, você é um exemplo de dedicação e companherismo. Nunca me esquecerei de você!

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Ainda que eu falasse as línguas dos homens e dos anjos, e não tivesse amor, seria como o metal que soa ou como o sino que tine.
E ainda que tivesse o dom de profecia, e conhecesse todos os mistérios e toda a ciência, e ainda que tivesse toda a fé, de maneira tal que transportasse os montes, e não tivesse amor, nada seria.
E ainda que distribuísse toda a minha fortuna para sustento dos pobres, e ainda que entregasse o meu corpo para ser queimado, e não tivesse amor, nada disso me aproveitaria.
O amor é sofredor, é benigno; o amor não é invejoso; o amor não trata com leviandade, não se ensoberbece.
Não se porta com indecência, não busca os seus interesses, não se irrita, não suspeita mal;
Não folga com a injustiça, mas folga com a verdade;
Tudo sofre, tudo crê, tudo espera, tudo suporta.

1 Coríntios 13:1-7

APRESENTAÇÃO

A tradicional medicina chinesa (TMC) tem ganhado destaque nesta última década na sociedade ocidental moderna e na comunidade científica. A Organização Mundial de Saúde (OMS), desde 1978, tem promovido a integração da TMC nos sistemas nacionais de cuidado à saúde; principalmente em países desenvolvidos. Os benefícios da TMC vão desde o tratamento da síndrome respiratória aguda severa até a depressão.

Fungos macromicetos, tradicionalmente utilizados pela TMC, têm sido alvos de estudos científicos. Tais estudos objetivam a comprovação documentada da sua ação terapêutica. Esta tese objetivou a avaliação das potencialidades farmacêuticas dos macromicetos *Cordyceps sinensis* e *Pleurotus ostreatus*, obtidos por fermentação submersa, quanto à ação hipolipidêmica, antiaterosclerótica, e protetor da disfunção puberal em modelos hiperlipídicos em ratos machos *Wistar*, bem como a ação antitumoral contra células do neuroblastoma do *Cordyceps sinensis*. Os resultados da pesquisa foram organizados em quatro capítulos com o intuito de informar os dados relevantes do estudo desenvolvido no período de 2010 a 2013.

Devido à forte presença dos modelos hiperlipidêmicos nos artigos, bem como a ação hipolipidêmica observada, o capítulo inicial tratou de uma revisão sobre os principais compostos hipolipidêmicos encontrados nos macromicetos, as estatinas. Os capítulos seguintes referem-se às atividades experimentais das novas propriedades medicinais dos cogumelos. Dentre os aspectos inéditos apresentados neste estudo estão o primeiro relato do efeito hipolipidêmico da biomassa de *Cordyceps sinensis* obtida por fermentação submersa em modelos hiperlipídicos crônicos, a contribuição do conceito referente à participação das doenças hepáticas crônicas sobre os níveis de testosterona, o efeito citotóxico do Cordyceps sinensis contra as células do neuroblastoma humano IMR-32, e finalmente, o efeito hipolipidêmico da biomassa de *Pleurotus ostreatus*.

APRESENTATION

Traditional medicine chinese (TCM) is regarded as a complementary and alternative practice outside China. The World Health Organization since 1978 has been included TCM in national health care systems, mainly in developed contries. In addition, it is recognized that TCM can be an important determinant in the treatment of certain diseases.

Macromycetes fungi, traditionally used by TCM, are being focused on in the international literature. These studies aimed to document such biological effects. This thesis aimed to evaluate the pharmaceutical potential of *Cordyceps sinensis* and *Pleurotus ostreatus* cultived by submerged fermentation as hypolipidemic, antiaterosclerotic and protector of low testosterone observed in hyperlipemic models of male *Wistar* rats. Further, it also aims to study the outcomes of the *Cordyceps sinensis* against neuroblastoma tumor cells. Research results are been organized by chapters during 2010-2013 experimental pratictice.

Due to strong emphasis on hyperlipemic models outlined in papers, as well as protective effects from these macromycetes against hyperlipidemia, the initial chapter was focused on review about statins – a compound found naturally in macromycetes. The following chapters refer to the experimental results about the new medicinal effects of mushrooms. This is the first time a *Cordyceps sinensis* biomass supplementation has been used to normalize the blood lipid and the low testosterone levels induced by high-fat diet, as well as this is the first time a hypolipidemic potential was reported by *Pleurotus ostreatus* by submerged fermentation. Further observations also contribute to validaty the current knowlege concerning the role played by chronic liver disease on lower testosterone levels and cytotoxicity activity against IMR-32 human neuroblastoma cells after treatment with *Cordyceps sinensis*.

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LIST OF SYMBOLS AND ABBREVIATIONS

APO	Apoliprotein
AST	Aspartate aminotransaminase
BHT	Butylated hydroxytoluene
BUN	Blood urea nitrogen
CETP	Cholesteryl ester transfer protein
CLE	Conjugated linoleic acid
CoA	Coenzyme-A
Cr	Creatinine
CSBS	*Cordyceps sinensis* biomass supplementation
DMEM	Dubelcco´s modified eagle medium
DN	Diabetic nephropathy
FDA	Food and drug administration
FOX	Ferrous ion oxidation xlynol orange
GH	Growth hormone
HDL	High low density
HDL-C	High low density cholesterol
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
H_2O_2	Hydrogen peroxide
ICR	Imprinting control region
IFN-γ	Interferon gama
IGF	Insulin-like growth factor
INSS	International Neuroblastoma Staging System
IMR-32	Human neuroblastoma cells
LDL	Low density lipoproteins
LDL-C	Low density lipoproteins cholesterol
LFA	Lymphocyte function-associated
MHC	Major histocompatibility class
ML-236B	Compactin or mevastatin
MTT	Thiazolyl blue tetrazolium bromide
NBT	Nitro blue tetrazolium

NO	Nitric oxide
O_2^-	Superoxide anion
RPMI	Roswell park memorial institute
SSF	Solid-state fermentation
TCM	Traditional medicine chinese
VLDL	Very low density lipoprotein

CHAPTER – I

MICROBIAL STATINS

Biotransformation of agro-industrial wastes: high value biochemicals. Satinder Kaur Brar, Carlos Ricardo Soccol and Gurpreet Singh Dhillon (ed.):
Section 2: Bioactive secondary metabolites

RESUMO

Este trabalho objetivou a avaliação das potencialidades farmacológicas dos macromicetos *Cordyceps sinensis* e *Pleurotus ostreatus*, obtidos por fermentação submersa, quanto à ação hipolipidêmica, antiaterosclerótica, e protetor da disfunção puberal em modelos hiperlipídicos de ratos machos *Wistar*, bem como a ação antitumoral do extrato aquoso de *Cordyceps sinensis* contra células do neuroblastoma humano. Para a instauração do modelo hiperlipídico, uma dieta comercial basal foi aditivada com gordura vegetal hidrogenada (6% p/p) e gordura de porco (14% p/p). Para as determinações bioquímicas, foram realizadas as dosagens do colesterol plasmático, lipoproteína de baixa densidade (LDL), atividade da aspartato transaminase, uréia e testosterona. A atividade antiaterosclerótica foi inferida pela determinação da ativação macrofágica: capacidade fagocítica, volume lisossomal e produção de íons (ânion superóxido, peróxido de hidrogênio e óxido nítrico). Os resultados indicaram que o *Cordyceps sinensis* diminuiu significativamente os parâmetros bioquímicos colesterol plasmático (37%), triglicerídeos (35%), LDL (40%), bem como normalizou os níveis de testosterona e restaurou a função hepática. Similarmente, o *Pleurotus ostreatus* também melhorou o perfil lipídico, bem como diminuiu a ativação macrofágica em aproximadamente 70%. Em adição, a ação antitumoral do *Cordyceps sinensis* contra células do neuroblastoma humano foi caracterizada por uma taxa de inibição de 20%. Sendo assim, estes cogumelos possivelmente não substituirão os tradicionais medicamentos já existentes para o tratamento destas patologias, mas poderão de forma valiosa complementá-los.

Palavras-chave: *Cordyceps sinensis*, *Pleurotus ostreatus*, ação hipolipidêmica, testosterona, ação antiaterosclerótica, neuroblastoma.

Microbial statins

Leandro. F. dos Santos, Júlio C. de Carvalho, Rosália Rubel, Carlos R. Soccol

Abstract:

Statins are a class of antihypercholesteremic (or cholesterol-lowering) drugs which act on the liver by reducing steroid biosynthesis by inhibiting the activity of HMG-CoA reductase, the enzyme responsible for the first step in the synthesis of cholesterol (and other biomolecules). This, in turn, causes the reduction of the concentration of LDL (low-density lipoproteins) associated with increased risk of coronary disease, stroke and heart attack. The market for statins is around 25-30 billion dollars, with synthetic compounds such as atorvastatin having a large market share. However, fermentation-based statins, such as lovastatin and pravastatin have a market share around 10%, while the semi-synthetic simvastatin has a 50% market. Extracts from *Aspergillus terreus* and *Nocardia autotrophica*, as well as raw biomass rich in statins from oyster mushroom or *Monascus* sp are also sources of natural statins. This chapter describes briefly the action of statins, the market for these drugs, the potential for new bio-based statins and the production process for lovastatin and pravastatin.

1. INTRODUCTION

Statins are a class of antihypercholesteremic (or cholesterol-lowering) drugs which act on the liver by reducing the biosynthesis of the steroid by inhibiting the activity of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, the enzyme responsible for the first step in the synthesis of cholesterol (and other biomolecules). Among statins, there are the molecules that are produced through synthetic means and those produced via fermentation-based processes with their semi-synthetic derivatives. The rational production of fermentation-based statins and discovery of new potential statin-producers is stimulated by the current market value, with a market share around 60%. Natural statins have a 2-3 billion dollar market (Bizukojc et al. 2007; Findlay et al. 2007; Morikawa et al. 2002; Rodger 2010; Rozman and Monostory 2010; Seraman et al. 2010; Vilches Ferrón et al. 2005; Weber et al. 2007), while the semi-synthetic simvastatin has a 50% market.

Among the advantages for fermentation-based statins, there is the possibility of utilization of agro-industrial by-products for sustainable bioproduction, and the possibility of using selected or modified microorganisms to obtain new statins. Statins also have additional biological effects, such as microvascular endothelial protection function (in the presence of hyperglycemia, thus inhibiting the early stage of diabetic microangiopath), or regression of fatty streak lesions (Arikan et al. 2012; Bizukojc et al. 2007; Nozue et al. 2012; Seraman et al. 2010; Vilches Ferrón et al. 2005).

All bioactive secondary metabolites are common on traditional therapeutic regimens for hyperlipidemia which have been associated with increased risk of coronary disease, stroke and heart attack. Hyperlipidemia is a leading cause of death in many countries, which is often triggered by hypercholesterolemia (the accumulation of cholesterol in the blood), leading to atherosclerosis (Talayero and Sacks 2011).

This chapter highlights some of the major hypolipidemic properties and aspects relevant to fermentation-based statins, such as mechanism of action, market value of these drugs, the potential for new bio-based statins and the production process for the most common fermentation-based statins. It will also present examples of well-characterized non-statin hypolipidemic agents.

2. HYPERLIPIDEMIA AND THE PROCESSES LEADING TO ATHEROSCLEROSIS

2.1 Definitions

Hyperlipidemia or dyslipidemia is a condition characterized by an increased concentration of lipids (triglycerides, cholesterol, or both) and lipoproteins (low density lipoprotein – LDL and very low density lipoprotein - VLDL) in the blood. Specific terms to increased blood concentrations of triglycerides are referred to as hypertriglyceridemia, while increased blood concentrations of cholesterol are referred to as hypercholesterolemia. The term hyperlipoproteinemia refers to increased blood concentrations of lipoproteins (Talayero and Sacks 2011).

2.2 Causes of hyperlipidemia and its association with atherosclerotic processes

The mechanisms behind pathologies must be understood in order to develop better drugs. The causes underlying hyperlipidemia and atherosclerosis are discussed in this section.

2.2.1 Causes of hyperlipidemia

Several causes have been reported to cause hyperlipidemia; high-fat diets, obesity, endocrine disorders (such as diabetes mellitus, hypothyroidism or hyperadrenocorticism), and cholestasis. Among these risk factors, high-fat diets are the main cause of hyperlipidemia. Higher amounts of saturated fat, trans-fat and cholesterol intake in high-fat diet cause increased LDL levels. However, other risk factors and LDL participation have a central role in the atherosclerotic development process.

A vast number of studies confirmed the intimate and causative relationships between dyslipidemias and diabetes (Subramanian and Chait 2012). In diabetes mellitus, there is deficiency of insulin. The chylomicrons and VLDL are released into blood and should be unloaded by lipases located on the vascular endothelium of tissues. However, these enzymes cannot function to its full extent because of the insulin deficiency, since insulin can regulate lipase gene expression (Bouraoui et al. 2012). In accordance with lipase inhibition, there will be increased triglycerides levels

or hypertriglyceridemia. High-triglyceride levels are markers for several types of atherogenic lipoproteins, especially apo C-III (Fukui et al. 2011).

Hypercholesterolemia has been stressed as one of the common biochemical findings in primary hypothyroidism. In general, hypothyroidism is associated with hypercholesterolemia mainly due to the elevation of total cholesterol and LDL-C levels, whereas there is an increase in triglyceride levels due to decreased activity of the lipoprotein lipase. There were multiple mechanisms accounting for atherosclerosis in patients with hypothyroidism, including hypercholesterolemia, insulin resistance, increased oxidation of LDL-C (overview of the atherogenesis bellow) (Arikan et al. 2012).

Comparative aspects of hyperadrenocorticism and arteriosclerosis have shown that advanced hyperadrenocorticism exhibit widespread arteriosclerosis with calcific complications. This disease shows a higher hypertriglyceridemia and hypercholesterolemia due to the associated metabolic alterations. Cortisol concentrations induce the insulin resistance and lipase activity (Ottosson et al. 1994; Wexler 1971).

Hypercholesterolemia always occurs in cholestasis, a disturbance of the secretion of bile salts by the liver, as bile is the chief pathway for the elimination of cholesterol from the body (Wagner et al. 2009).

2.2.2 Overview of the atherogenesis

Atherosclerosis has been reported as a result of hyperlipidemia and it is regarded as a lipid-induced inflammatory disease where the immune system plays a pivotal role in its initiation and progression. In general, atherogenesis begins at sites of endothelial injury caused by smoking, infection, diabetes mellitus, hypertension and which results a nidus for monocyte and lipid/lipoprotein accumulation into the underlying arterial intima (Gu et al. 2012). Lipid and lipoprotein accumulation is due to adhesiveness and permeability of the endothelial injury. As LDL accumulates, it may be entrapped extracellularly in arteries, thus being subjected to a milieu conducive to various kinds of enzymatic and chemical modification (oxidation and glycation). Lipoproteins undergo minimal oxidation during circulation, but become progressively oxidized within the arterial wall. The monocytes subsequently differentiate into macrophages, ingest modified - LDL and other particles (Koga and

Aikawa 2012). In a way, scavenger receptors of macrophages bind oxidized LDL but not native LDL, leading to development of cholesterol ester-engorged cells or foam cells, the precursors of atherosclerotic lesions. T-cells infiltrating into the endothelial lesion may recognize antigenic signals presented by the activated macrophages and generate an immune response by inflammatory cytokines and growth factors production which stimulate smooth muscle cell migration and proliferation (Kzhyshkowska et al. 2012). The smooth muscle cells, in turn, secrete extracellular matrix components that form a fibrous cap over the underlying atherosclerotic lesion. Foam cells undergo apoptosis and release their lipid contents to form an extracellular lipid core that is contained under the fibrous cap. All this process will result in thickened intima-media and atherosclerosis plaque development in arteries. Moreover, in general, atherosclerotic plaques with thin fibrous cap, large lipid cores, and numerous macrophages are most likely to rupture. Thus, once plaques ruptures, the thrombogenic contents are exposed to platelets and coagulation factors in circulating blood, initiating a thrombosis. Thrombosis is a blood clot within a vessel, obstructing or stopping the flow of blood (Steinbrecher 1999; Østerud and BjØrklid 2003).

Atherosclerosis usually does not cause signs and symptoms until it severely narrows or totally blocks a vessel. Therefore, atherosclerosis represents the world´s largest problem from modernity by taking 17.1 million lives a year. According to the most recent data available, one in five deaths in developing countries is due to atherosclerosis in its various forms (Ellis et al. 2010; Messner and Bernhard 2010).

Treatments for atherosclerosis may include lifestyle change, surgery and medicines, such as antihypertensives (angiotensin-converting enzyme inhibitors, calcium channel blockers, thiazide diuretics), antiplatelets and mainly statins against high cholesterol and LDL levels (Borshch et al. 2012).

The mechanisms discussed about the model of atherogenesis initiation outlined above are mainly based on experimental animal models with high rate of developing lesions, i.e., genetically hyperlipidemic animals and fat-fed. However, there is supporting evidence that many molecules, cytokines, growth factors, scavenger receptors, etc, are expressed and produced similarly in humans, validating this whole mechanism and opening several strategies for the development of new treatments.

3. THE STATIN-BASED THERAPEUTIC STRATEGIES

3.1 General characteristics of statins

Statins are oral inhibitors of the 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which are well-established agents to lower cholesterol levels and prevent cardiovascular morbidity and mortality. HMG-CoA reductase is the rate-limiting enzyme that catalyzes the conversion of HMG-CoA to L-mevalonate, a key pathway for cholesterol biosynthesis. Therefore, statins are the first-line of defense in drug treatment of hypercholesterolemia (Morikawa et al. 2002; Rozman and Monostory 2010; Weber et al. 2007).

3.2 Chemical structure and mode of action - Statins

Statins have differences in their chemical structure that could translate into different pharmacological properties and pharmacokinetic parameters (bioavailability, half-life, protein binding, metabolism and excretion routes, lipophilicity). All statins have a structural component which is similar to HMG portion of HMG-CoA (Fig. 1 2). Thus, statins act as competitive inhibitors of the HMG-CoA reductase. In general, competitive inhibition of HMG-CoA reductase by statins decreases the conversion of HMG-CoA to mevalonate, the rate limiting step of cholesterol endogenous synthesis (Fig. 1 1). The reduction of downstream metabolic intermediates leads to increased expression of LDL-receptor on the surface of hepatocytes and so increase uptake of LDL-C from the circulation (Campo and Carvalho 2007; Nirogi et al. 2007). Aside from their cholesterol-lowering effects statins can reduce coenzyme Q10 and dolichol. Coenzyme Q10 is a mitochondrial coenzyme which is essential for the production of ATP and immune system while the role of the dolichol remain elusive (Cantagrel et al. 2010; Kumar et al. 2009).

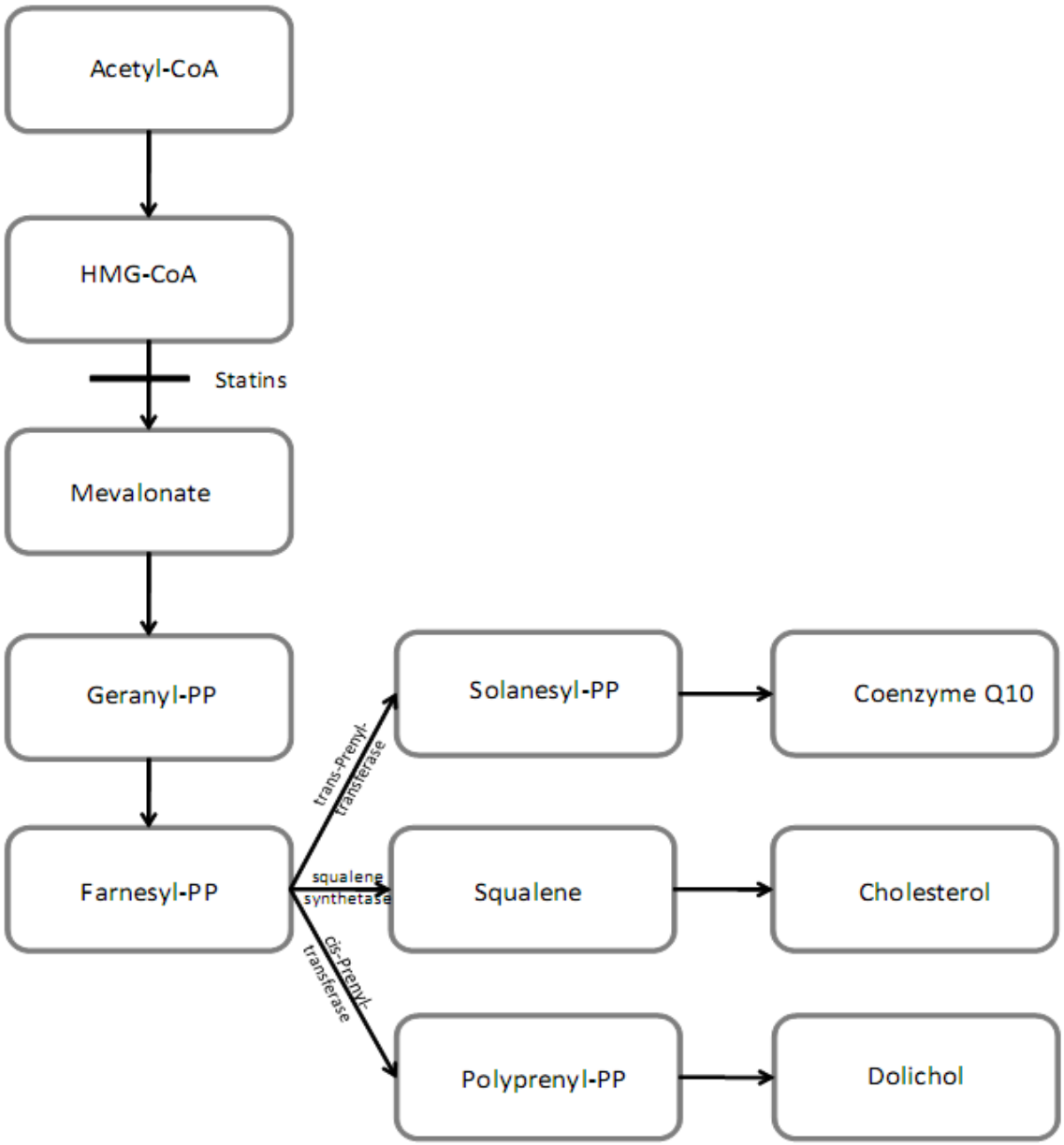

Figure 1 1 – Statins: mechanism of action. Statins act as competitive inhibitors of the HMG-CoA reductase. Thereby, statins decreases the conversion of HMG-CoA to mevalonate

Currently, seven statins are used in medical practice: lovastatin, pravastatin, simvastatin, fluvastatin, atorvastatin, rosuvastatin and pitavastatin. From these, the last five are synthesized chemically while lovastatin and pravastatin are derived from fungal fermentation. Chemical structures of statins are shown in Fig. 1 2. The recurrent structure in all statins is a hydroxyl carboxylic acid which mimics HMG-CoA. The lactonized forms of statins are converted to the active hydroxy acids in the liver.

The efficiency with which statins are absorbed and inhibit the synthesis of cholesterol are affected by their structure, leading to a wide heterogeneity of traditional therapeutic regimens used by statins, as well as its pharmacological properties. Table 1 shows the therapeutic doses and half-lives for statins.

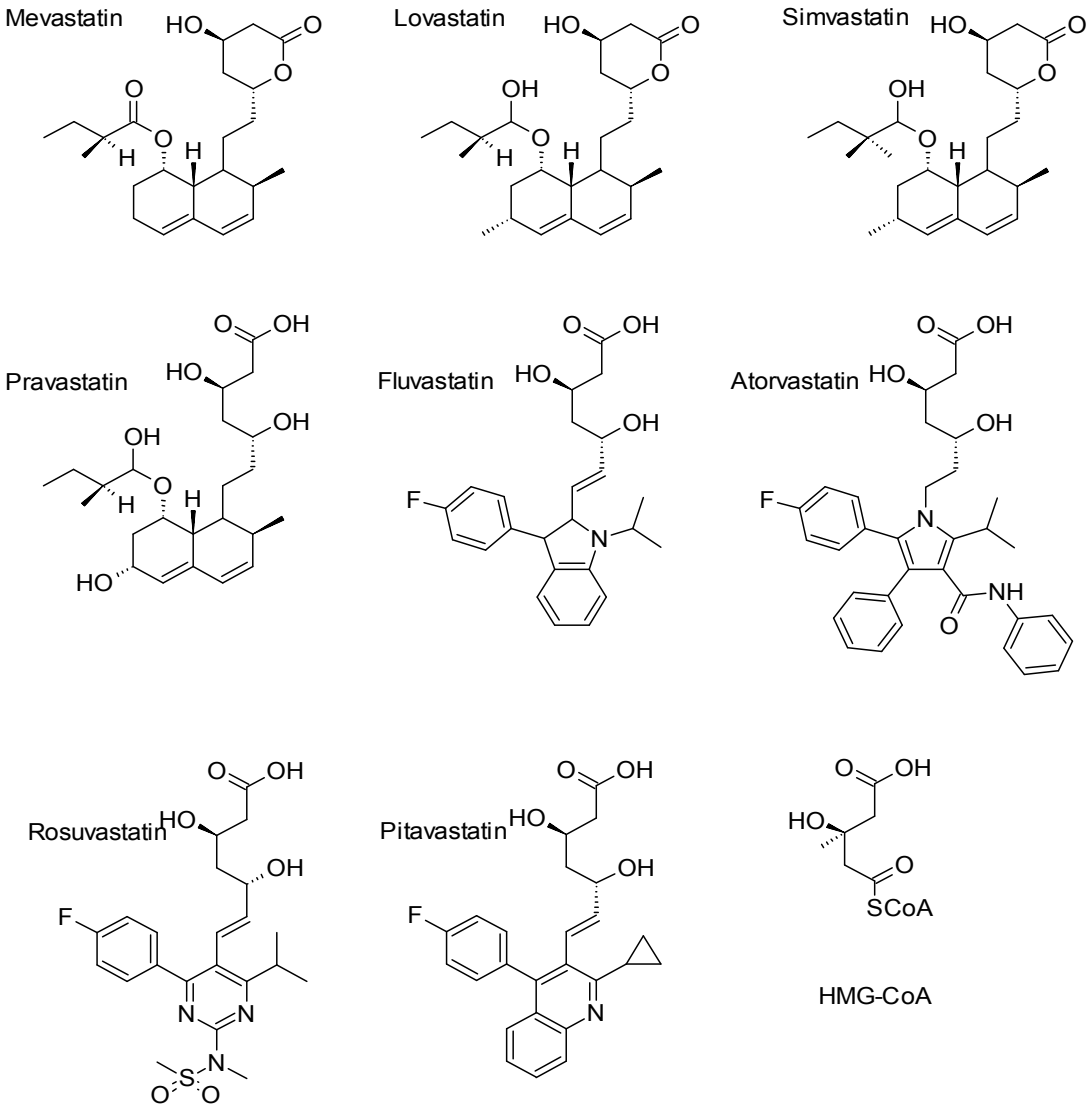

Figure 1 2 – Chemical structure of the statins used clinically

Table 1 - Traditional therapeutic regimens used by statins and its pharmacological properties (Betteridge 2010; Catapano 2010; Jones et al. 2003; Knopp 1999)

Statins	Therapeutic dose *	Elimination half-life (h)	Solubility
Fluvastatin	20-80 mg	1-2	Lipophilic
Atorvastatin	10-80 mg	14	Lipophilic
Rosuvastatin	5–40 mg	19	Hydrophilic
Pitavastatin	2-4 mg	11-18	Moderately Lipophilic
Simvastatin	5-80 mg	1-2	Lipophilic
Lovastatin	10-80 mg	3	Lipophilic
Pravastatin	10-80 mg	1-2	Hydrophilic

* Dose required for inhibition of 50% HMG-CoA reductase

3.3 Other relevant effects of statins

Aside from their cholesterol-lowering effects statins are known to have a range of effects, such as vasodilatation, effects on coagulation, inflammatory response modulation, atherosclerotic plaque regression and immunomodulatory effects.

Endothelium-ameliorating effects of statin therapy were observed in patients with symptomatic heart failure. The beneficial effect of statin therapy on endothelium-dependent vasodilatation in patients was associated with coenzyme Q_{10} reductions. Therefore, statins can increase nitric oxide (NO) bioactivity, which is consistent with enhanced endothelium function and reduce synthesis of proinflammatory proteins on the endothelial cell surface, which may reduce inflammation (Brull et al. 2001; Gajendragadkar et al. 2009; Koh et al. 2004; Strey et al. 2005).

Statins may affect the expression levels of genes involved in coagulation, such as thrombomodulin and nitric oxide syntase-3. Thrombomodulin is a transmembranous glycoprotein and plays an important role in the anti-coagulant system as the receptor of thrombin leading to accelerated protein C activation (Gajendragadkar et al. 2009; Morikawa et al. 2002).

Statins still have been demonstrated to inhibit superantigen-induced T cell activation, MHC class II antigen down-regulation, LFA-1 binding, reduced heart and kidney transplant rejection, reduced mortality with staphylococcal bacteremia and reduction of high-sensitive C-reactive protein in patients with coronary artery disease (Fehr et al. 2004; Weber et al. 2007).

3.4 *Current market situation of statins – The billion dollars drugs*

The beginning of the 21st century brought an economic growth reduction for most advanced countries, with an average around 2% per year. Some economies even shrinked, and overall growth in Europe in 2012 was stagnant at 0.2%. Despite economic losses, statins´s market was substantially maintained. Accounting for 6.5 % of the total pharmaceutical market share, statin drugs are the most widely sold drugs in history. To date, drug companies are earning $ 26 billion in annual sale (Fig. 1 3) – a single class of drugs producing more profits than Google - and are expected to continue to increase in the years ahead. The first statin, lovastatin, was launched in America in the 1980s with revenues of U$ 200 million (Findlay et al. 2007; Rodger Murphree 2010). The statin market (Fig. 1 3) is still on the rise, although the growth is decelerating after early 2007. While new drugs are developed and the older patents expire, statin therapies may become even more accessible, and the market may be expected to reach U$35 billion by 2023.

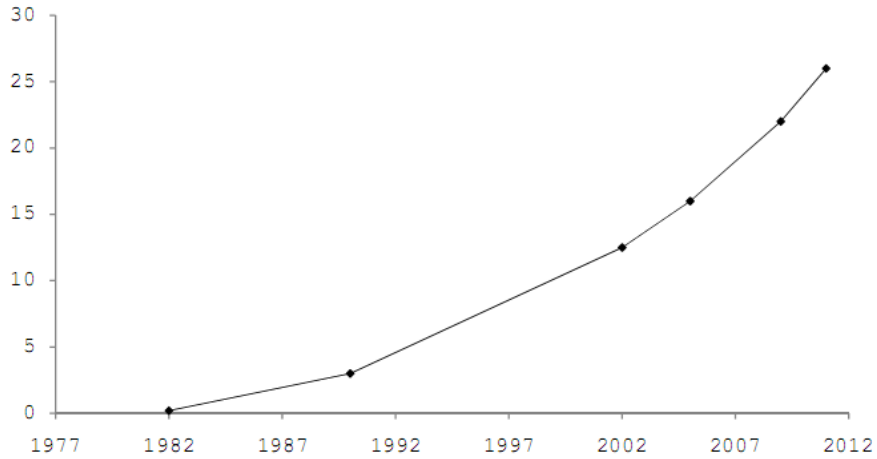

Figure 1 3 - Statin market (in billions). Source: Information derived by *Consumer Reports Best Buy Drugs – The statin drugs, The Telegraph* and *Forbes magazine*

Statin use has increased in recent years as hyperlipidemia is being diagnosed more frequently. There is more evidence supporting the hazard of high levels of lipids in the blood, and consumers have become increasingly aware of beneficial effect of statins. New prescriptions of statins show a market share around 10% for bio-based compounds (lovastatin and pravastatin) (Findlay et al. 2007), and a 50% market for the semi-synthetic simvastatin. This panorama is probably due to the recent expiration of patents rights for lovastatin (2001) and simvastatin (2006) and the entry of generics. The expiration of atorvastatin patents (2012) will probably bring it back as a generic statin. On the other side, with new drugs in the pipeline and an annual growth of emerging markets (such as Latin America), the bio-based statin market is sure to remain huge.

4. BIO-BASED STATINS: PRODUCTION PROCESS AND POTENTIAL FOR NEW SUBSTANCES

Although most of the newer statins are synthetic, bio-based statins are of interest because its production skips several synthetic steps, providing a high value-added molecule from low cost substrates in an eco-friendly process.

ML-236B, known as compactin or mevastatin, was the first breakthrough in efforts to find a hypolipidemic agent by Akira Endo at 1976. By 1980, Merck had discovered lovastatin which has been shown to be chemically very similar to mevastatin, differing by one methyl group (absent in mevastatin). Endo also had

discovered the same compound, lovastatin (Brown and Goldstein 2004). Soon after the development of lovastatin, several screening efforts led to the development of similar molecules. Bio-based statins biosynthetic pathway starts from acetyl and malonyl-CoA units linked to each other (Auclair et al. 2001; Barrios-González and Miranda 2010; Komagata et al. 1989).

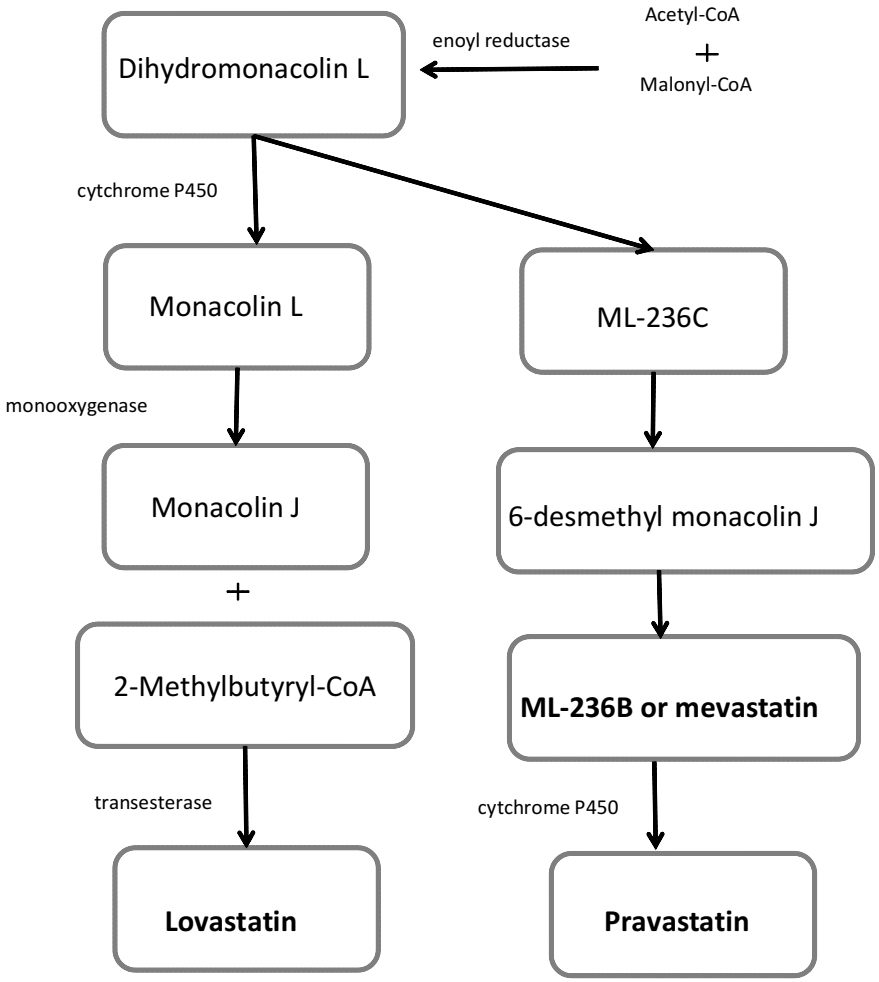

Figure 1 4 - Bio-based statins biosynthetic pathway

4.1 Lovastatin

4.1.1 General

Lovastatin is a fungal secondary metabolite discovered in the seventies and introduced in the American market in the 1980s as Mevacor by Merck. It was the first statin to be approved by FDA and it was formerly called as mevinolin or monacolin K.

Lovastatin is administered as β-hydroxi lactone which over the time converts *in vivo* to the respective hydroxy acid form, partly similar to HMG-CoA. The hydroxyl acid form is a weak acid (pK_a = 4.31) with a molar mass of 422.55 while its lactone form has a higher (pK_a=13.5) (Bizukojc et al. 2007; Brown and Goldstein 2004; Lisec et al. 2012; Seenivas et al. 2008; Seraman et al. 2010).

4.1.2 *Currrent and potential uses of lovastatin*

Besides its cholesterol-lowering properties, lovastatin has been reported as a potential therapeutic agent for the treatment of various types of cancer. Recent *in vitro* studies have shown that lovastatin inhibits proliferation of anaplastic thyroid cancer cells through up-regulation of p27 by interfering with the Rho/ROCK (serine/threonine kinase Rho Kinase) mediated pathway – this pathway has been suggested to be involved in the regulation of cancer cell motility. Other studies have shown endothelial protection function of lovastatin in the presence of hyperglycemia. Endothelial dysfunction, such as decreased endothelium-dependent vasorelation plays a key role in the pathogenesis of diabetic vascular disease. Lovastatin was able to improve mesenteric responses to acetylcholine (Gajendragadkar et al. 2009; Zhong et al. 2011). Oxidative stress has been linked to the cause of many human diseases, such as heart failure, coronary artery and chronic kidney disease and neurodegenerative disturbances. It arises from an imbalance between an excessive generation of reactive oxygen species, reactive nitrogen species and insufficiency of antioxidant agents. Oral administration of lovastatin has been demonstrated to reduce oxidative stress and change the activities of antioxidant enzymes (Kumar et al. 2011).

4.1.3 Production process

4.1.3.1 Potential producers

Lovastatin is produced by a variety of filamentous fungi. Some of the important microbial sources are *Monascus sp*., *Penicillium sp. and Aspergillus sp*. Species were found to be the most significant producers of lovastatin, such as *Monascus purpureus, Monascus ruber, Aspergillus terreus, Aspergillus flavipes* found

in the literature. Table 2 lists some species evaluated or developed for lovastatin production. Although several species produce low amounts of lovastatin, they are shown in order to illustrate the genera variability.

Table 2 - Lovastatin production (in mg/L of fermented broth) by selected strains reported.

Microorganism	**Strain**	**mg/L**	**Reference**
Aspergillus terreus	ATCC 20542	100	Porcel et al. 2008
Penicillium citrinum	MTCC 1256	589	Ahmad et al. 2010
Aspergillus terreus	Isolate	400	Szakács et al. 1998
Aspergillus terreus	DRCC 122 (uv mutant)	2200	Kumar et al. 2000
Aspergillus terreus	Isolate	400	Samiee et al. 2003
Monascus pilosus	MK-1 (a mutant strain)	725	Miyake et al. 2006
Monascus purpureus	MTCC 369	351	Sayyad et al. 2007
Biospora sp.	Isolate	13	Osman et al. 2011
Cylindrocarpon radicicola	Isolate	7.1	Osman et al. 2011
Penicillium spinulosum	Isolate	15.8	Osman et al. 2011
Trichoderma viridae	Isolate	36	Osman et al. 2011
Mycelia sterilia	Isolate	15.3	Osman et al. 2011
Aspergillus terreus	DSM 13596	310	Benedetti et al. 2002

New rapid screening methods were developed to find new potential producers based on the activity of lovastatin against the yeast *Candida albicans.* In this method, the diameter of the inhibition zones (obtained on plates of *Candida albicans*) correlated linearly with the quantity of lovastatin impregnated in the paper disc (Bizukojc et al. 2007; Seraman et al. 2010; Vilches-Ferrón et al. 2005). Other method for detecting lovastatin-producing strain is based on PCR for specific genes related to lovastatin synthesis, detecting suitable strains more quickly and effectively (Kim et al. 2011).

4.1.3.2 Fermentation

Submerged fermentation and solid-state fermentation (SSF) have been used for lovastatin production. Large-scale processes were developed using *Aspergillus terreus* in submerged fermentation. Enhanced strategies, such as the use of antibiotics, cultivation in fed-batch mode, and medium development led to higher yields (Jia et al. 2010; Porcel et al., 2008; Seenivas et al. 2008).

Solid-state fermentation is a potential alternative to produce lovastatin which generates less effluent and uses less power. SSF uses various solid substrates, such as besan flour, barley, sago, long grain rice (all these substrates yield high lovastatin production, > 110 mg/g dry substrate); mixed solids can also be used to formulate economical substrates for commercial production (Subhagar et al. 2009; Valera et al. 2005). The inocula for these processes may be either a liquid culture or a spore suspension; after inoculation, the fermenters are maintained at a temperature, pH and aeration rate which is characteristic of each strain for several days. Typical values are 28^{o}C, pH 6.5, 1.5 vvm and 7 days for *Aspergillus terreus* strains (Bizukojc et al. 2007).

4.1.3.3 Culture medium characteristics

As with any fermentation product, the culture medium has a significant effect on the rate of production and yield of lovastatin. The type of carbon source (e.g. fructose, lactose, glycerol), nitrogen source (e.g. soybean meal, corn steep liquor, yeast extract) and the C:N mass ratio used in the medium influenced production of lovastatin and microbial biomass by *A. terreus.* The results have shown that the presence of excess carbon (slowly metabolizable carbon source) under nitrogen limitation greatly enhanced the rate of production of lovastatin. Nitrogen limitation diverts more carbon to lovastatin metabolic pathways (Casas López et al. 2003). Most liquid culture media use glucose as the main carbon source, but some authors suggest that this sugar strongly represses lovastatin synthesis (Miyake et al. 2006), which explains why continuous or fed-batch processes enhance the yield. In addition to glucose, several culture media also use starches and complex mixed sources, such as oatmeal, soybean meal, peptones and yeast extract in the culture medium (Lisec et al. 2012).

A variety of mineral nutrients is also added to the culture media, usually K_2HPO_4, $MgSO_4$, and ammonia, urea or nitrates. Microelements are seldom added, being provided by the complex nutrients used (Lisec et al. 2012).

Studies have shown that the supplementation of the culture medium with B-group vitamins enhances lovastatin synthesis by *Aspergillus terreus*. The hypothesis that the synthesis of lovastatin requires a high throughput of coenzymes, thus the application of its precursors in the form of B-group vitamins would give a positive effect on lovastatin production (Bizukojc et al. 2007). Impact of other supplements, such as linoleic acid has demonstrated that micromolar concentrations of this fatty acid enhances lovastatin yield. Possibly, early supplementation of linoleic acid anticipates the production of oxylipins, thus mimicking the critical cell mass necessary for the onset of lovastatin production (Sorrentino et al. 2010). Vegetable oils stand out as a promising substrate as an additional carbon source for lovastatin production (Sripalakit et al. 2011).

4.1.3.4 Downstream processing of lovastatin

As lovastatin has a very low polarity, the concentration from the culture broth may be carried out by liquid-liquid extraction. However, there is a substantial portion of intracellular lovastatin (Benedetti et al. 2002), which may not be easily extracted. In addition, the molecule may be oxidized if not properly processed, leading to hard to separate impurities–antioxidants or inert atmospheres should be used. After extraction and concentration, the statin is usually purified by crystallization, although chromatography and ion exchange steps may also be used. The final compound should have a purity of at least 99.5%. Figure 1 5 illustrates a possible lovastatin downstream process.

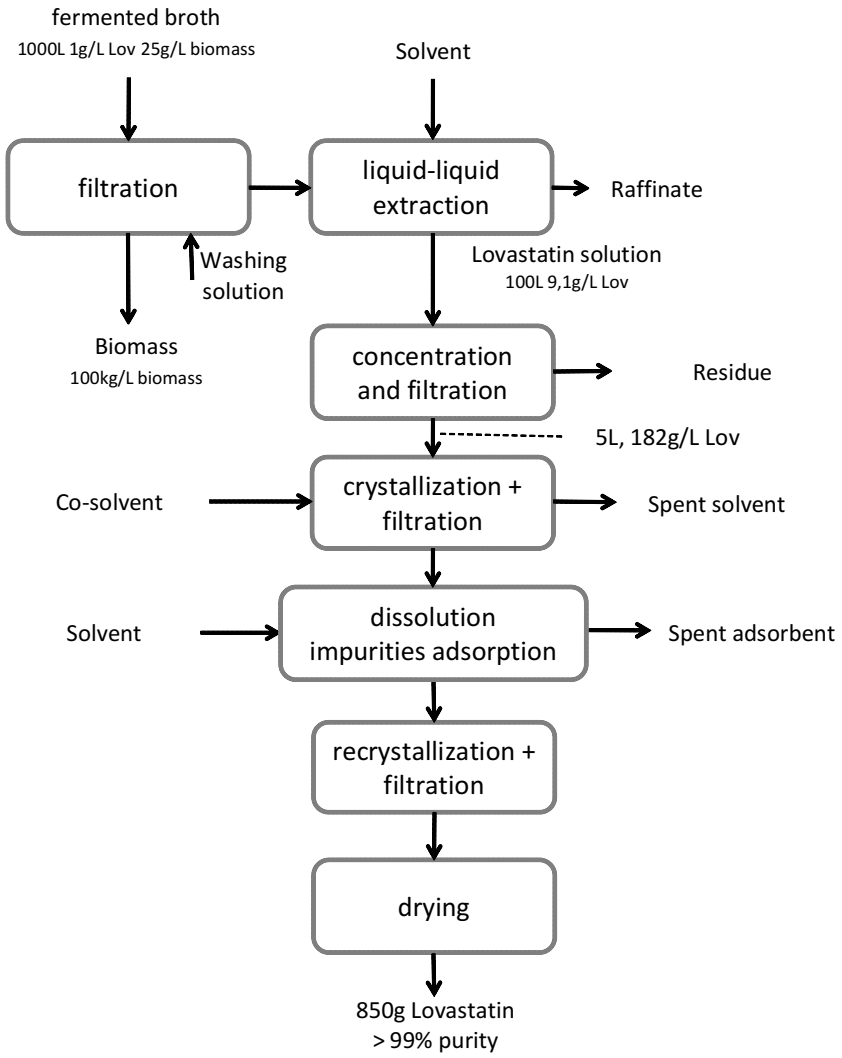

Figure 1 5 - Lovastatin downstream processing relying on crystallization operations

4.1.4 Simvastatin production (derivatization of lovastatin)

The natural product lovastatin can be derived in its analog semi-synthetic simvastatin. Substitution of the α-methylbutyrate side chain with α-dimethylbutyrate is most effective in treating hypercholesterolemia, while lowering undesirable side-effects. An alternative method for the simvastatin synthesis is a selective enzymatic deacylation of lovastatin. Due to the effectiveness of simvastatin, numerous multistep synthesis from lovastatin to simvastatin have been described in the patent literature. For example, a process for preparing simvastatin from lovastatin or mevinolinic acid in salt form comprises treating either starting material with cyclopropyl or butyl amine. In another process, lovastatin was hydrolyzed to acid form and then isolated in the

form of amine salt like cyclopropyl or t-octylpropyl amines. The salts isolated were directly methylated without any protection or deprotection of hydroxyl groups. Then, simvastatin ammonium salts were converted to simvastatin by conventional methods of lactonization (Kumar et al. 1998; Vaid and Narula 2006).

4.2 Pravastatin

4.2.1 General

Pravastatin was discovered as a bioactive metabolite of mevastatin, in efforts to develop new statins. It was launched on the market in 1991 (Li 2009). Pravastatin as well as the other statins exists in two forms, lactone and open-ring hydroxy acid form (active form); besides the open ring, pravastatin has an extra hydroxil group in comparison with lovastatin, being hydrosoluble. Pravastatin is more effective than lovastatin in moderate doses. In human plasma, pravastatin can be determined by enzyme immunoassay or in human urine by HPLC and UV detection (Darwish et al. 2009; Whigan et al. 1989).

4.2.2 Current and potential uses of pravastatin

Neuroprotection by pravastatin in acute ischemic stroke has been demonstrated in rats even when given after stroke onset. Among the mechanisms responsible for improved neurological outcome is the inhibition the release of potentially damaging cytokines such as interleucine-6 in the early phase of cerebral ischemia (Berger et al. 2008). Other potential uses of pravastatin are in diabetic nephropathy. Diabetic nephropathy (DN) is the principal cause of end-stage renal failure in the Western world and leads to major mortality. DN is characterized by endothelial dysfunction following a variety of proinflammatory insults. Studies have provided evidence of the protective properties of pravastatin through an up-regulation in endothelial constitutive nitric oxide synthase expression in diabetic group (Casey et al. 2005).

4.2.3 Production process

4.2.3.1 Overview

Pravastatin is obtained by two-step fermentation, firstly mevastatin is produced by *Penicilium citrinum* or other potential producer such as *Streptomyces carbophilus* and converted by hydroxylation of mevastatin to form pravastatin. The hydroxylation of mevastatin in *Streptomyces carbophilus* is catalyzed by a $CytP450_{sca}$ monooxygenase system (Sakaki 2012; Serizawa 1996). Recent advances in the molecular characterization of the $CytP450_{sca}$ and their responsiveness to mevastatin have been achieved. For example, molecular approaches for transcriptional regulation of the cytochrome $P450_{sca}$ from *Streptomyces carbophilus* by mevastatin sodium salt or cloning, characterization and expression of the gene encoding $CytP450_{sca}$ from *Streptomyces carbophilus* involved in production of pravastatin. $CytP450_{sca}$ DNA sequences have been annotated in GenBank® (Sakaki 2012; Watanabe et al. 1995; Watanabe and Serizawa 1998). A new strain of *Streptomyces flavidovirens* has been used to produce pravastatin (Gururaja et al. 2003). A *Monascus ruber* strain is capable to produce 3000mg/L of pravastatin in a short fermentation time, according to Benedetti *et al.* (2002). In relation to bioconversion, it has been established that actinomycetes could hydroxylate mevastatin to pravastatin. The degree of conversion by cells was 65-78% of mevastatin added and 65-88% of mevastatin taken up (Peng and Demain 2000).

4.2.3.2 Downstream aspects

Pravastatin fermentation is followed by isolation and purification. A method of isolating and purifying pravastatin or its pharmaceutically salt involves a step of extracting using an organic solvent, such as ethyl acetate (Nobunari et al. 2003). Other studies have reported purification methods which use high-performance liquid chromatography (HPLC) (Haytko and Wildman 1992). Some polymorphs of pravastatin sodium have been described as obtainable from a process wherein aprotic and protic solvent are used (Pater and Wnukowski 2012). The fact that pravastatin is hydrophylic has the benefit of limiting the contamination by lypophilic compounds in the initial step of the process and the control of solvent extraction by

broth acidification, but may lead to partial dimerization in the concentration steps of the solvent extract.

Traces of mevastatin can still be present in the end product after lyophilization to remove solvent. Mevastatin and pravastatin are structurally closely related. Thus, purification of pravastatin is tedious but important for the production of a safe and efficient drug. Methods have been proposed for the extraction of pravastatin and the concomitant removal of impurities. It has been found that a rate of solution containing pravastatin:mevastatin has increased when is added water-immiscible solvent (e.g isopropyl acetate, methyl isobutyl ketone, etc) with water or an aqueous solution at a pH value ranging from 5.0 to 6.5 (Johannes et al. 2009). Mevastatin can also be highly toxic to microorganisms responsible for biotransformation, especially to mould fungi, thus mevastatin concentration must only be maintained at a low level during industrial production (Minquan et al. 2006).

Despite known pravastatin production process, still there are problems that need to be solved. During fermentation steps, there is a common problem characterized by degradation of pravastatin (e.g. hydrolysis of pravastatin) resulting in loss of product. This phenomenon also can occur with lovastatin. Therefore, use of specific nitrogen or carbon sources to avoid statin hydrolysis during fermentation, as well as deletion of genes encoding enzymes that hydrolyze statins are necessary (Klaassen et al. 2009). During work-up procedures, unwanted loss of product occurs as result of lactonization leading to pravastatin lactone. Any breakthrough approach suppressing lactonization is therefore of great relevance in productive process. Elevated temperatures, certain pH-regimes and traces of other molecules can promote unwanted lactonization. Aprotic solvents have been appointed to suppress lactonization in pravastatin sodium downstream process (Pater and Wnukowski 2012).

5. PERSPECTIVES OF THE NATURAL NON-STATIN HYPOLIPIDEMIC AGENTS

High statin doses are often associated with the increased frequency of adverse effects. In addition, all statins have been observed to cause myopathy, and the risk of adverse effects on muscle increases with the use of high doses (Riphagen et al. 2012). Therefore, non-statin hypolipidemic drugs can be an alternative treatment option. There are several promising novel therapeutic approaches for the treatment of hyperlipidemia and atherosclerosis based on non-statin hypolipidemic agents which are expected to be of great benefit for patients with adverse effects. Some of these agents may be produced using bioprocesses.

5.1 Niacin

Niacin, also known as vitamin B_3 or nicotinic acid, at levels higher than the required vitamin dose, it functions as a vitamin, favourably affecting atherogenic lipoprotein release, as well as decrease cholesterol, triglycerides, LDL, VLDL levels and increase HDL-cholesterol levels. Niacin acts on rasing apoAI and lowering apoB. These apoliproteins are the main protein components of circulating HLD (apoAI) and of LDL and VLDL (apoB). However, the absolute magnitude of these lipid-modifying effects is highly dependent not only on the daily dose, but also on the lipid phenotype at baseline (Chapman et al. 2010; Rozman and Monostory 2010).

5.2 Ezetimibe

Ezetimibe, a cholesterol-absorption inhibitor, acts on cholesterol from diet and does not affect the absorption of fat-soluble vitamins, triglycerides or bile acids. The effect of ezetimibe on the progression of atherosclerosis remains unknown. It selectively inhibits cholesterol absorption by binding to the Niemann-Pick C1-like 1 (NPN1L1) protein. Combined therapy with ezetimibe and a statin provides an incremental reduction in LDL-C levels of 12 to 19% (Rozman and Monostory 2010; Teramoto et al. 2012).

5.3 Cholesteryl Ester Transfer Protein (CETP) inhibitors

CETP promotes the transfers of cholesteryl esters from antiatherogenic HDLs to proatherogenic apoB - principle protein components of circulating LDL and VLDL. Moreover, it facilitates the transport of triglycerides between the lipoproteins. Thus, CETP transfers lipids from one lipoprotein to another that results in equilibration of lipids between lipoprotein fractions. A deficiency of CETP is associated with increased HDL levels and decreased LDL levels, supporting the therapeutic potential of CETP inhibition as an approach to retarding atherogenesis (Rozman and Monostory 2010; Tzotzas et al. 2011).

5.4 Fibrates

Fibrates are generally effective in lowering elevated plasma trycerides and cholesterol. It is a class of amphipatic carboxylic acids. Fibrates can implicate five major mechanisms underlying the modulation of lipids: a) induction of lipoprotein lipolysis; b) induction of hepatic fatty acid uptake and reduction of hepatic triglyceride production; c) increased removal of LDL particles; d) reduction in neutral lipid exchange between VLDL and HDL and e) increase in HDL production and stimulation of reverse cholesterol transport. In general, fibrates are considered to be well tolerated, with an excellent safety profile (Rozman and Monostory 2010; Saha and Arora 2011).

6. PERSPECTIVES

It is well known that downstream processes can be considered one of the main factors involved in the final cost of the product as it can includes multiple steps such as filtration, extraction, chromatography, crystallization, adsorption flow through and drying (Straathof 2011; Winkelnkemper et al. 2011). Thus, the use of fungal biomass, recognized as safe by FDA/OMS, can be an interesting alternative in controlling lipid profile and avoid a complex downstream process (Tab. 3). There are already reports investigating the hypolipidemic effect of fungal biomasses (Santos et al. 2012). The support of this idea comes from herbal medicines which basically use plants in raw state and produce therapeutic action.

Table -3 - Hypocholesterolemic effect of fungal biomass or its fractions on several animal models

Microorganism	**Animal model**	**Amount/ fraction**	**Reference**
Auricularia auricula	ICR mice	Ethanol extract 150 mg/kg/d b.w.	Chen et al. 2011
Cordyceps sinensis	ICR mice	Hot-water extract from mycelia 150 and 300mg/kg/d,	Koh et al. 2003
Coriolus versicolor	Rats	Water extract	Hor et al. 2011
Pleurotus ostreatus	Wistar rats	20% biom/ feed	Santos et al. 2012
Pleurotus ostreatus	Rabbits	10% dried fruit/ feed	Bobek and Galbavy 1999
Pleurotus ostreatus	Humans	30 g dried fruit/ soup	Schneider et al. 2011

The use of statins has radically improved the therapy of coronary disease since 1990. The huge market for these molecules means that constant effort is being pursued by pharmaceutical companies and research institutes for developing new and more efficient molecules. Although semi-synthetic derivatives will likely dominate

the market in the near future, microorganism screening may lead to the development of new bio-based statins. On the other side, traditional foods are being thoroughly studied in order to elucidate other cholesterol-lowering or synergistic mechanisms. This opens the path for the development of novel nutraceutical foods and dietary supplements.

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CHAPTER – II

Cordyceps sinensis BIOMASS PRODUCED BY SUBMERGED FERMENTATION IN HIGH-FAT DIET FEED RATS NORMALIZES THE BLOOD LIPID AND THE LOW TESTOSTERONE INDUCED BY DIET

ACCEPTED LETTER

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Cordyceps sinensis BIOMASS PRODUCED BY SUBMERGED FERMENTATION IN HIGH-FAT DIET FEED RATS NORMALIZES THE BLOOD LIPID AND THE LOW TESTOSTERONE INDUCED BY DIET

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ABSTRACT

This study investigated the effect of *Cordyceps sinensis* (LPB VI) biomass supplementation obtained from submerged fermentation on blood lipid and low testosterone induced by high-fat diet (HFD). The experiments were carried out using a long-term intake of HFD and HFD plus simvastatin or *C. sinensis* (4 months). Our results show that plasma cholesterol, triglycerides and LDL were decreased by *Cordyceps sinensis* biomass supplementation (CSBS). A long-term intake of HFD caused a significant liver damage which has been reverted by CSBS. CSBS normalized decreasing testosterone levels observed in high-fat diet feed rats. All these findings lead us to suggest that *C. sinensis* was able to decrease blood lipid concentration, increase hepatoprotective activity and normalize testosterone levels.

Key words: *Cordyceps sinensis*, high-fat diet, hypolipidemic effect, testosterone.

INTRODUCTION

The biological properties of *C. sinensis* mycelium, including anti-inflammatory and anti-tumor activities, have previously been investigated *(*Liu et al., 2011; Yan et al*.,* 2011).

Interestingly, in addition to their mycelium effects obtained from solid-state fermentation, *C. sinensis* biomass obtained from submerged fermentation (Chimilovski et al., 2011) could be an effective agent in lipid metabolism. This hypothesis was supported by some evidences about glucan isolated from the *C. sinensis* (Wu et al., 2005, 2007). Remarkably, hypocholesterolemic effects of glucans have been shown in various studies (Tiwari and Cummins, 2011; Lei et al., 2012). Similarly, submerged fermentation is a conventional approach to produce high quality biomass and this technique possesses particular advantages such as superiority in process control and easy recovery of biomass (Sun and Xu, 2009).

Current interest in the effect of glucans on lipid metabolism main is centered on the possibility that the glucans could entrap bile acids in the intestine and thus increase bile acid exclusion in the feces (Bowles et al., 1996). Second, cholesterol uptake could be inhibited by glucan in the intestinal wall (Drozdowski et al., 2010). Third, glucans undergo a fermentation process to produce short-chain fatty acids that can inhibit cholesterol synthesis (Drozdowski et al., 2010; Turunen et al., 2011).

Atherosclerosis, the complex interaction of macrophages with serum cholesterol in arterial wall, is the leading cause of cardiovascular disease worldwide and it has become a serious social problem. Among various factors leading to atherosclerosis, high low-density lipoprotein cholesterol, triglycerides and total cholesterol have been considered to be the major risk factors in its pathogenesis (Østerud and BjØrklid, 2003; Ding et al., 2012; Zha et al., 2012).

Since increased cholesterol and other lipid parameters may affect the development of atherosclerosis and cholesterol buildup in the coronary arteries (Zha et al., 2012), we assumed that hypolipidemic activity must be studied in *C. sinensis* biomass obtained from submerged fermentation.

Current knowledge concerning the role played by chronic liver disease on testosterone levels has been demonstrated. Studies have shown that low testosterone levels are common in men with severe liver disease (Grossmann et al., 2012). The pathogenesis of low testosterone levels in men with chronic liver disease involves dysregulation of the hypothalamo-pituitary-gonadal axis at multiple levels (Grossmann et al., 2012). Thereby, the severity of chronic liver disease could become obviously a serious problem for boys in pubertal stage.

Here we investigated whether a long-term intake of fat diet could decrease testosterone levels through liver damage (caused by liver-fat deposition) and the beneficial effects of CSBS, produced by submerged fermentation, on hyperlipidemia pattern and low testosterone observed in the high-fat diet feed rats.

EXPERIMENTAL

Diet preparation

The modified basal diet used was a modification of the laboratory animal feed (Labina, Purina®, São Paulo, Brazil) with the following ingredients (g/100g): lard, 14 and hydrogenated vegetable fat, 6. To prepare it, pulverized standard diet and melted lipids (lard and hydrogenated vegetable fat) were mixed. A daily average of the food intake was determined by adding the food consumed each day by all of the rats of each group and dividing it by the number of rats per group.

Study design

All procedures involving animals were approved by the Positivo University Committee for Animal Welfare. Forty male *Wistar* rats, 30 days weighing 110 g (10 ± g) were divided into four groups (ten per group). The animals were kept in the animal house at a temperature of 24 ±2 °C with a 12/12 hour light/dark cycle for 4 months and fed with the respective diets and water *ad libitum.* Control group was fed with basal diet without modification, HDF and HFD + simvastatin (Medley, Campinas–SP, Brazil) or *C. sinensis* groups were fed with modified basal diet and modified basal diet + simvastatin or *C. sinensis* respectively. When required, simvastatin and *C. sinensis biomass* were added together with modified basal diet.

The dosage of drug and biomass were 10.36 mg/Kg and 10% (w/w) (drug or biomass/feed) for a total of 14 weeks respectively.

Biochemical determinations

At the end of the experiments, the animals were anesthetized through ethereal inhalation, and blood samples were collected through cardiac puncture for measuring the plasma cholesterol, triglycerides, LDL, AST activity, urea and testosterone.

The plasma lipid, urea and creatinine measurements were performed in an ADVIA 1650 automated system (Bayer AG, Leverkusen, Germany). Testosterone measurements were performed by direct immunoassay on the Roche E170 system.

Liver lipid hydroperoxides

Dosage of lipid hydroperoxides was carried out on methanolic extract of liver tissue as described by Nourooz-Zadeh et al. 1994. A 300 mg portion of the liver right lobe (laparoscopic liver resection) was homogenized in 1 mL methanol, using an electric homogenizer (GGS 27, Bosch). After centrifugation (5000 g, 5 min, 4°C), a 50 µL aliquot of the supernatant (cell-free extract) was stored for further measurement of the total proteins, and 90 µL aliquots were disposed into six centrifuge vials (1,5 mL). To three of these vials, 10 µL of methanolic 10 mM triphenylphosphine was added, thereby generating three blanks. To the other three vials, 10 µL of methanol was added. All the six vials were vortexed and then incubated for 30 min at room temperature. After that, 900 µL FOX 2 (100mM xylenol orange, 4 mM BHT, 25 mM sulfuric acid and 250 mM ammonium ferrous sulfate, 90% methanol, 10% ultrapure water) was added to all vials. After mixing, the samples were incubated for another 30 min at room temperature. The absorbance was measured at 560 nm using a spectrophotometer (Ultrospoc 2000, Pharmacia Biotech). The results were corrected for the extract protein concentration.

Liver total proteins

The method described by Bradford was carried out for this measurement (Bradford, 1976). Briefly, 250 µL Bradford reagent was added to 10 µL of cell-free extract in a microplate. After 5 min at room temperature, absorbance at 595 nm was measured using a microplate spectrophotometer (Benchmark, Bio-Rad). Protein concentration was determined interpolating absorbance values in a standard curve generated by known concentrations of bovine serum albumin.

Histopathology and staining

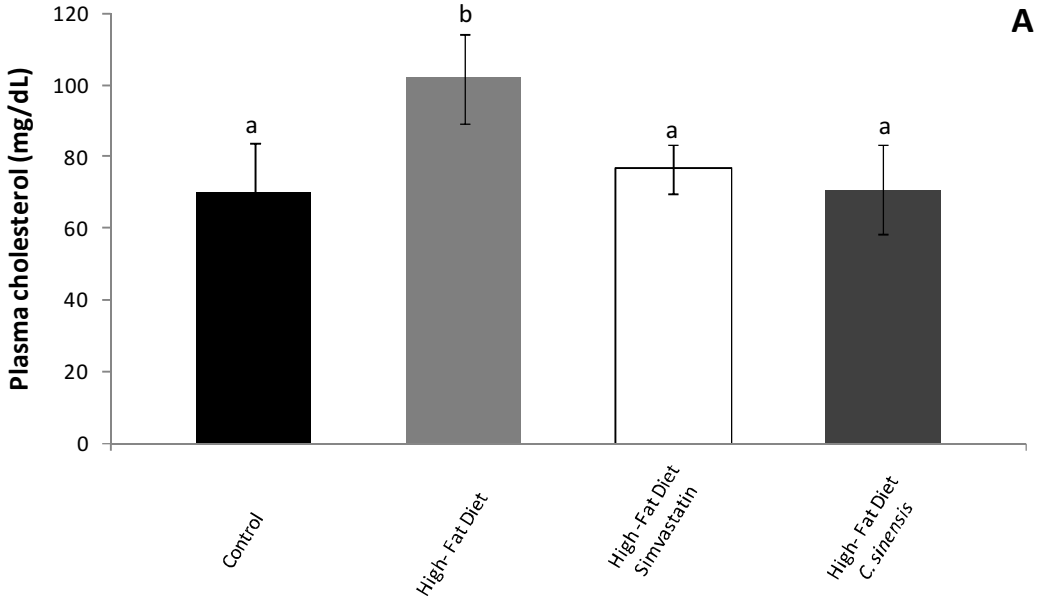
We performed biopsies of the livers of animals from different experimental groups: (A) Control, (B) HFD and (C) HFD plus *C. sinensis*. The biopsy specimens was fixed in formalin, embedded in paraffin, and cut in cryostat with serial sections of 3 to 6 µm after freezing. Thereafter, they were stained with Sudan-black.

Statistical analysis

The data are presented as mean ± SEM values. Statistical analysis was performed by one-way ANOVA, followed by the Tukey test. The value of $p<.05$ was taken to indicate statistical significance.

RESULTS

Fig. 2 1 shows the lipid parameters (plasma cholesterol, triglycerides and LDL) of rats fed HFD and HFD supplemented with simvastatin and biomass (*C. sinensis).* In the diet supplementation experiments levels of lipid parameters were calculated using enzymatic-colorimetric method. High-fat diet feed rats showed an increase in plasma cholesterol and LDL levels; however, the triglycerides levels exhibited no changes compared with control animals. Simvastatim and *C. sinensis* administered to high-fat diet feed rats as a diet supplement was well tolerated and caused positive response (significant decrease) such as plasma cholesterol (37%), triglycerides (35%) and LDL levels (40%). Interestingly, simvastatin (synthetic hypolipidemic) and *C. sinensis* showed similar trends.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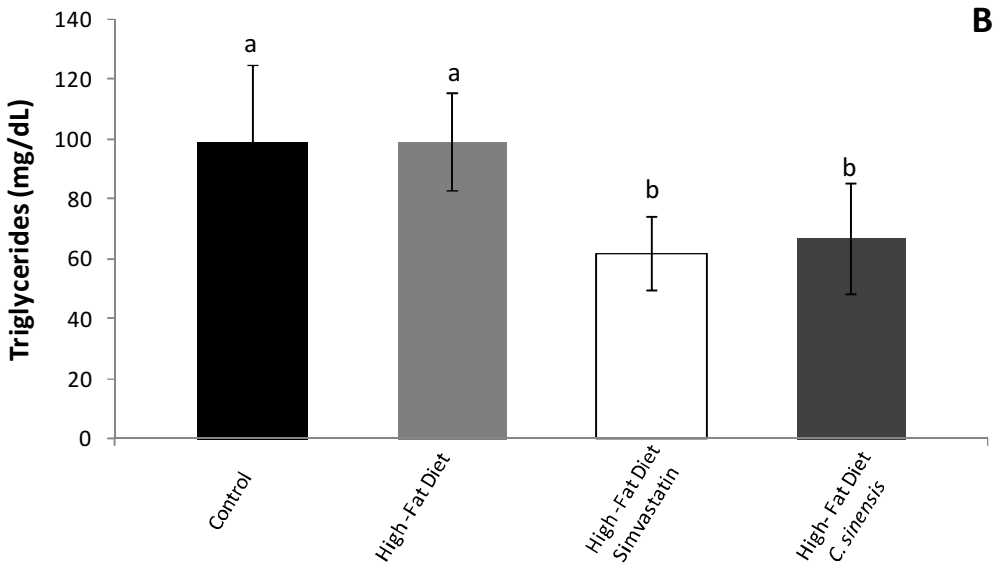
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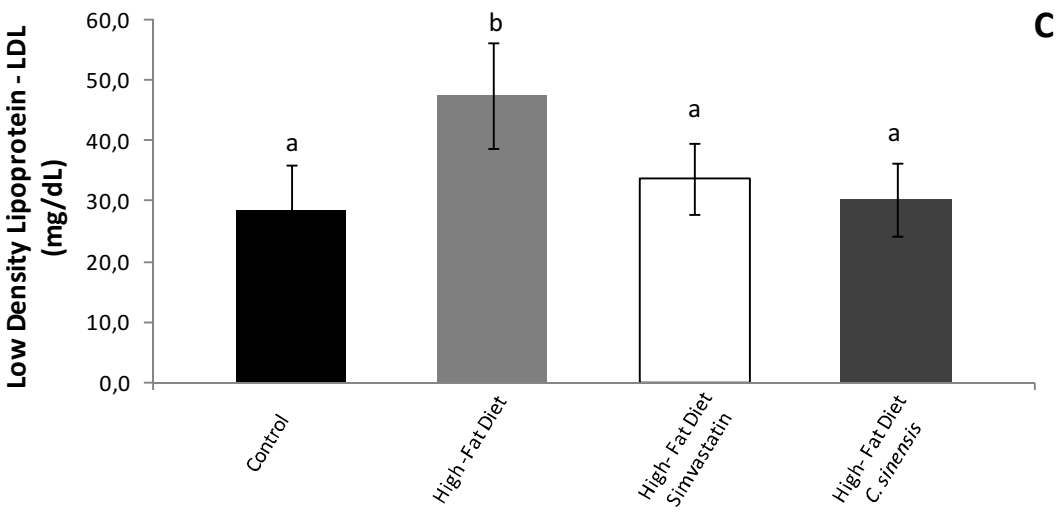


Figure 2 1 - Plasma cholesterol, triglycerides and LDL in rats fed HFD and HFD supplemented with simvastatin and biomass (*C. sinensis).* Data are mean ± SEM values of ten rats per treatment group. (A,B,C) ^{b} p<.05 compared to control group.

From the HFD group, the diet increased liver-fat deposition (Fig. 2 2), which remained significantly higher than in control group at the end of the experiment. In contrast, rats treated with *C. sinensis* showed a lower liver-fat deposition. Fig. 2 2 shows liver cryosections stained with Sudan black for specific labeling of lipids. As shown in Fig. 2 2, lipids accumulate predominantly in HFD group, while in HFD plus *C. sinensis* is spared. Aspartate aminotrasferases were lower in the treated groups than those of the HFD group (Fig. 2 3A). Plasma urea in the HFD group or HFD plus treatment was decreased compared with control group (Fig. 2 3B). Measurement of liver plasma hydroperoxide concentrations were realized by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine (Nourooz-Zadeh et al., 1994). Liver plasma hydroperoxides of treated groups were lower than those of the HFD group (Fig. 2 3C).

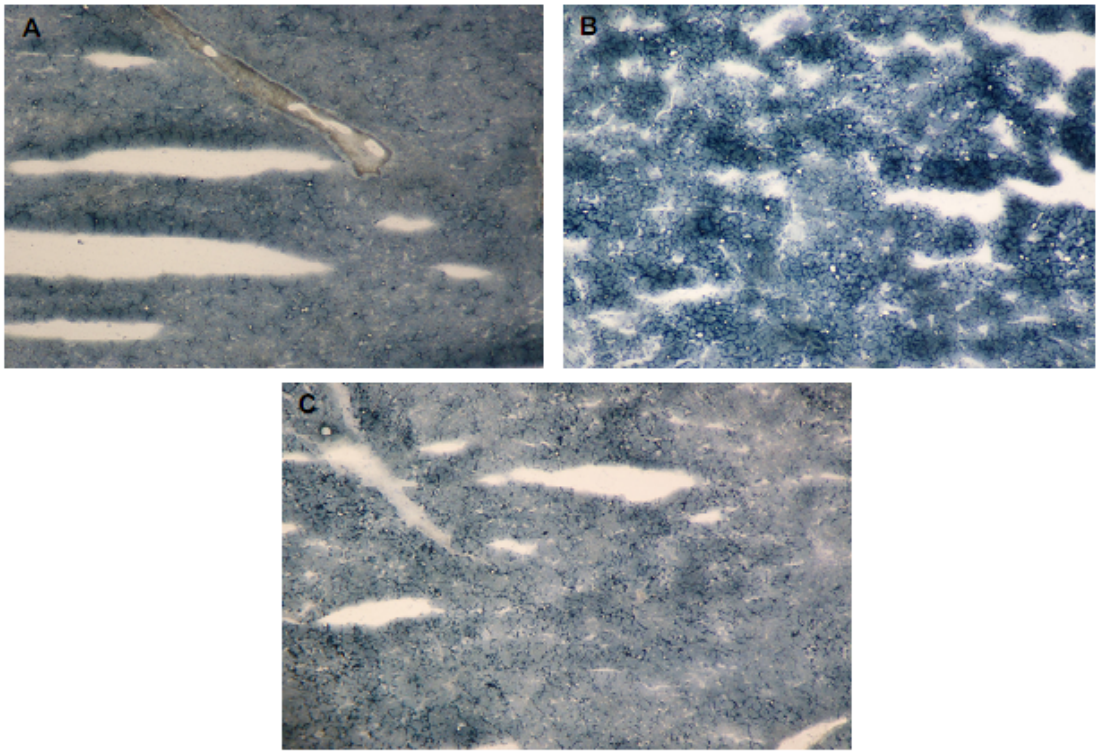
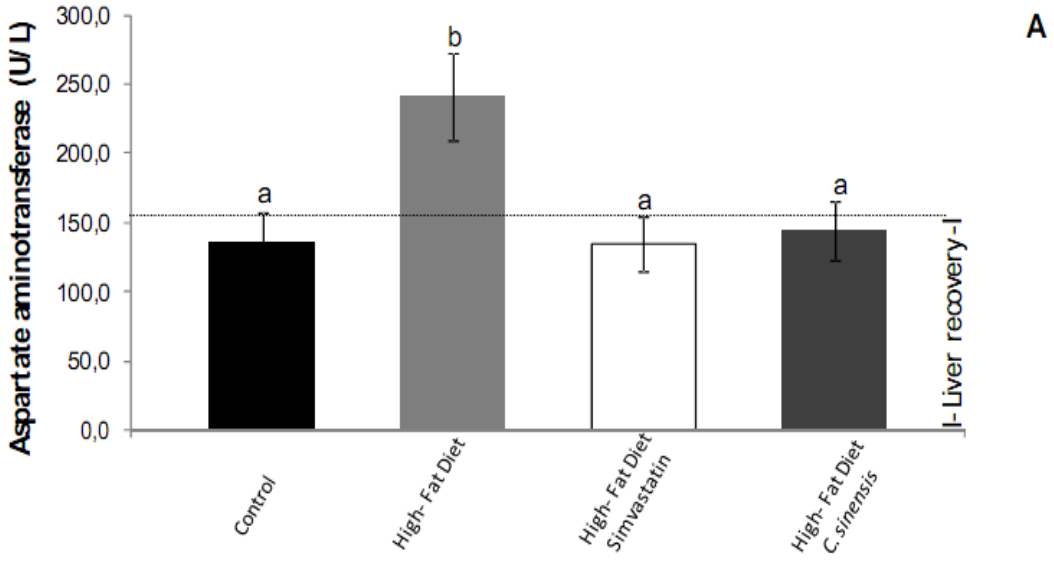


Figure 2 2 - Livers of animals from different experimental groups: (A) Control, (B) HFD and (C) HFD plus *C. sinensis.* Histopathological analysis (Sudan black, 10x magnification). Dark spots represents the accumulation of lipid droples.



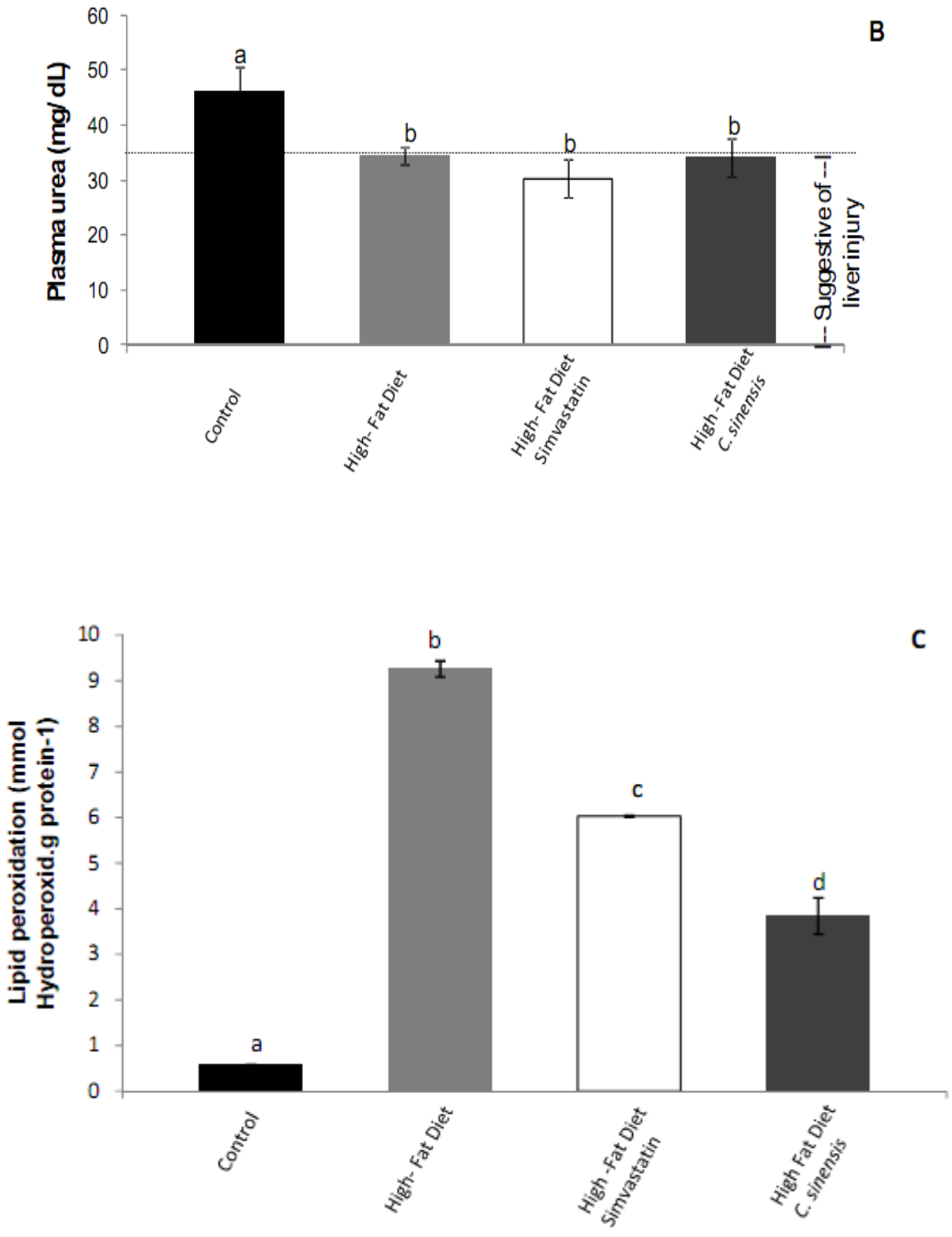


Figure 2 3 - Effect of HFD on aspartate aminotransferase, plasma urea and lipid peroxidation. Data are mean ± SEM values of ten rats per treatment group. ^{b,c,d} $p<.05$ compared to control group. Horizontal lines represent groups with similar mean.

The results of this study show how repeated HFD (able to increase lipid parameters), without increasing body weight (data not shown), results in lower testosterone levels and possibly promote the delayed onset of signs of pubertal

maturation in male rats. Interestingly, plasma testosterone was identical for control and treated groups (Fig. 2 4).

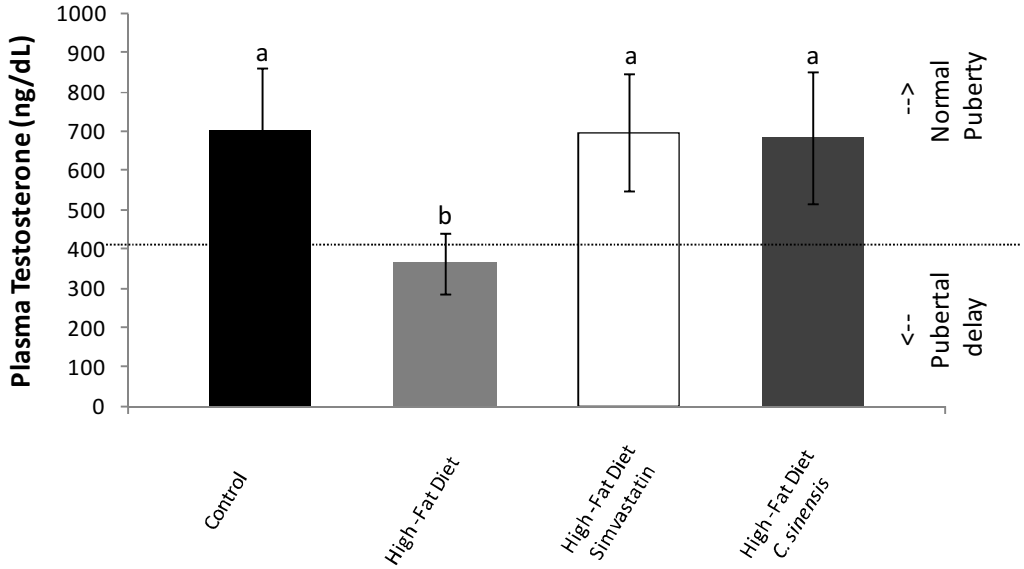


Figure 2 4 - Effect of HFD on plasma testosterone. Data are mean ± SEM values of ten rats per treatment group.[b] p<.05 compared to control group.

DISCUSSION

This study investigated whether a long-term intake of fat diet supplemented with *C. sinensis* biomass, produced by submerged fermentation, given to rats would modify the hyperlipidemia pattern and low testosterone observed in the high-fat diet feed rats.

The findings in this study confirm that *C. sinensis* biomass in this experimental conditions is sufficient to decrease lipid parameters such as plasma cholesterol, triglycerides and LDL (Fig. 2 1). Notably, simvastatin (synthetic hypolipidemic) and *C. sinensis* showed similar trends, which may reflect their potential use against hyperlipidemia and atherosclerosis. It is possible that compounds (lovastatin, glucans, steroids, niacin, CETP inhibitors and cordycepin) derived from the cell body *C. sinensis* may be the actives hypolipidemic materials (Wu et al., 2005, 2007; Rozman and Monostory, 2010; Leu et al., 2011; Tiwari and Cummins, 2011; Lei et al., 2012). In addition, all statins have been observed to cause myopathy, and the risk of adverse effects on muscle increases with the use

of high doses (Rallidis et al., 2012). Therefore, *C. sinensis* can be an alternative treatment option as far as it could decrease the doses of statins used in therapeutic regimes. One reason can be devised for invariable levels of triglycerides in high-fat fed rats: our experimental diet was prepared with laboratory animal feed enriched with commercial hydrogenated lard which contained high concentrations of conjugated linoleic acid (CLA). Notably, hypotriglyceridemia effect of CLA has been shown in various studies (Andreoli et al., 2009; Shu-Chiun et al., 2012). Thus, CLA could alleviate hypertriglyceridemia caused by HFD. At any rate, the treated groups had a positive effect on triglycerides levels (Fig. 2 1B).

Diet conditions showed liver damage by accumulation of lipid droplets, which may reflect hepatic steatosis (Fig. 2 2) (Amacher, 2011). All other analyses also showed liver damage such as aspartate aminotransferase activity, plasma urea and lipid peroxidation (HFD group) (Fig. 2 3). From the treated groups, plasma urea did not show improved levels. Despite that simvastatin and *C. sinensis* could be considered biologically inactive against liver damage when plasma urea levels are observed (Fig. 2 3B), it is possible that treatment period was not able to restore urea cycle enzymes. However, aspartate aminotransferase activity, liver histopathology analyses and lipid peroxidation demonstrated that treated groups, especially in the HFD plus *C. sinensis*, exhibited hepatoprotective activity (Fig. 2 2 and Fig. 2 3A, C). The assessment of oxidative damage by the lipid peroxidation in tissue exposed to oxidative stress has been proposed to assess tissue damage (Wang et al., 2012). Correlation between HFD and improved oxidative stress was reported (Chaudhari et al., 2012).

An interesting effect was observed in the plasma testosterone of animals from HFD group (Fig. 2 4). HFD promoted lower testosterone levels which probably could delay onset of signs of pubertal maturation in male rats. Insight into the relationship between HFD and lower testosterone levels may be provided by at least two reasons: (a) dysregulation of the hypothalamo-pituitary-gonadal axis at multiple levels since liver damage (caused by HFD) was observed in our study - low testosterone levels in men with chronic liver disease have been demonstrated (Grossmann et al., 2012), (b) liver disease could effectively decrease the insulin-like growth factor 1 (IGF-1) production. The IGF-1 is a single chain polypeptides consisting of 70 amino acids and regulated by liver. The liver is the central organ

of the endocrine growth hormone/insulin-like growth factor (GH/IGF) axis. The IGF system is involved in all aspects of male reproductive physiology and it increases during the onset of puberty (Caregaro et al., 1997; Flores et al., 1998; Lackey et al., 2000; Donaghy et al., 2002; Yoon et al., 2011). Still on reproductive function, we believe that the negative effects of high-fat diet feed rats on testosterone levels showed in our study may be due to no weight gain demonstrated by animals under this experimental conditions (data not showed) since the weight gain could be correlated with the higher leptin levels which suggest higher testosterone levels or precocious puberty (Terasawa et al., 2012; Wagner et al., 2012). The beneficial effect of *C. sinensis* on testosterone level disorders in HFD rats may be provided by liver recovery and the regulation of steroidogenesis by *C. sinensis* in rats Leydig cells (Hsu et al., 2003; Huang et al., 2004; Wong et al., 2007).

CONCLUSIONS

The results suggest that *C. sinensis* biomass supplementation in high-fat diet feed rats for 4 months normalizes the blood lipid and the low testosterone levels induced by HFD. Probably, *C. sinensis* biomass supplementation cannot replace the use of currently available drug regiments for lipid reduction, but can complement them. They may also enable the use of lower doses of therapeutic drugs, thereby decreasing the risk of dose-related side effects. Further observations also contribute to validity the current knowledge concerning the role played by chronic liver disease on lower testosterone levels. Further studies should be made in order to evaluate IGF levels, as well as other pubertal parameters to assess the delayed onset of signs of pubertal maturation.

ACKNOWLEDGMENTS

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DECLARATION OF INTEREST

The authors report no declaration of interest.

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CHAPTER – III

HYPOLIPIDEMIC AND ANTIATHEROSCLEROTIC POTENTIAL OF *Pleurotus ostreatus* CULTIVED BY SUBMERGED FERMENTATION IN HIGH-FAT DIET FED RATS

ACCEPTED LETTER

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Biotechnology and Bioprocess Engineering

Dear Mr. Freire dos Santos,

I am pleased to tell you that your work has now been accepted for publication in Biotechnology and Bioprocess Engineering.

It was accepted on 23-08-2012.

Thank you for submitting your work to this journal.

With kind regards

Prof. Sunghoon Park
Editor-in-Chief
Biotechnology and Bioprocess Engineering

HYPOLIPIDEMIC AND ANTIATHEROSCLEROTIC POTENTIAL OF *Pleurotus ostreatus* CULTIVED BY SUBMERGED FERMENTATION IN HIGH-FAT DIET FED RATS

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ABSTRACT

The ability of *Pleurotus ostreatus* (LPB 09) biomass cultived by submerged fermentation to produce beneficial effect on lipid profile and macrophages activity during a high-fat diet (HFD) for a long-term intake was investigated. Blood samples were collected through cardiac puncture for measuring the plasma cholesterol, triglycerides, low-density protein (LDL), high-density protein (HDL), aspartate aminotransferase (AST) activity, urea – blood urea nitrogen (BUN)/ creatinine ratio of rats fed on a HFD for 4 months. Dosage of lipid hydroperoxides was carried out on methanolic extract of liver tissue. Peritoneal macrophages activity was evaluated in relation to the superoxide anion, hydrogen peroxide and nitric oxide production, phagocytosis and lysosomal volume. The administration of *P. ostreatus* significantly altered the lipid profile and oxidative stress as related to the LDL and triglycerides decrease and inhibitory effects on superoxide anion and hydrogen peroxide production. All findings of this study lead us to suggest that the *P. ostreatus* could be an beneficial agent in hyperlipidemia and atherosclerosis treatments.

Keywords: *Pleurotus ostreatus*, submerged fermentation, hypolipidemic effect, antiatherosclerotic effect, high-fat diet.

INTRODUCTION

Pleurotus ostreatus have an important place among the commercially employed basidiomycetes because they have, nutritional, gastronomic and biological (medicinal) properties [1, 2]. Among its functions in organisms, *P. ostreatus* has been demonstrated biological properties including the antitumor activity, colitis-related colon carcinogenesis and immunomodulating effects [3–5].

Dried *P. ostreatus,* produced by solid-state fermentation and which are believed to contain a natural lovastatin-like compound, have been shown to provide significant cholesterol reductions in animals models and humans [6, 7]. These statins (lovastatin) are used as 3-hydroxy-3methylglutaryl coenzyme A (HMG CoA) reductase inhibitors and can treat hyperlipidemia [1]. Hyperlipidemia refers to increased concentrations of lipids (triglycerides, cholesterol, low density lipoprotein - LDL) in the blood. Notably, a high level of circulating lipid in the blood is one of the risk factors for the development of coronary atherosclerotic lesions [8–11].

Earlier study with *P. ostreatus* have suggested that submerged fermentation play an importante role in production of bioactive metabolites [12]. Submerged fermentation, or liquid-state fermentation, is a technique where a liquid medium and highly processed ingredients are used and a faster alternative method to obtain quality biomass [13, 14]. Interestingly, comparative studies between solid-state fermentation and submerged fermentation showed different levels of bioactive compounds and biological activities obtained in these thechniques [15, 16].

In view of the importance of the hypolipidemic properties of this mushroom in solid-state fermentation and differences demonstrated by comparative studies between solid-state fermentation and submerged fermentation, it is suggested that hypolipidemic activity must be studied in submerged fermentation or liquid-state fermentation.

Similarly, earlier studies have shown that macrophages play a part in pathogenesis of atherosclerotic lesions, oxidizing LDL and transforming themselves in foam cells and producing reactive oxygen species that may also oxidize LDL. In fact, there are several receptors for oxidized – LDL, but uptake of this oxidize LDL by macrophages through scavenger receptors leads to massive lipid accumulation inside the macrophage. In a way, scavenger receptors binds oxidize LDL but not native LDL, leading to development of cholesterol ester-engorged cells or foam cells,

the precursors of atherosclerotic lesions [9, 17–19]. Thus, considering the direct effects of reactive oxygen species on LDL in pathogenesis of atherosclerotic lesions, it is assumed that peritoneal macrophages activity must be evaluated as potentially ideal indicators to reveal the antiatherosclerotic potential.

The present study has addressed the outcome of *P. ostreatus* biomass obtained from submerged fermentation on hypolipidemic and atherosclerotic activity in rats fed on a HFD for a long-term intake (4 months).

MATERIALS AND METHODS

Diet preparation

The modified basal diet used was a modification of the laboratory animal feed (Labina, Purina®, São Paulo, Brazil) with the following ingredients (g/100g): lard, 14 and hydrogenated vegetable fat, 6. When required, *P. ostreatus* (LPB 09) biomass cultived by submerged fermentation was added together with modified basal diet. The dosage of biomass was 10% (w/w).

Study design

All procedures involving animals were approved by the Positivo University Committee for Animal Welfare. Forty male *Wistar* rats, 30 days weighing 110 g (10 ± g) were divided into four groups (ten per group). The animals were kept in the animal house at a temperature of 24 ±2 °C with a 12/12 hour light/dark cycle for 4 months and fed with the respective diets and water *ad libitum.* Control group was fed with basal diet without modification, HFD and HFD + simvastatin groups were fed with modified basal diet and modified basal diet + simvastatin (Medley, Campinas–SP, Brazil) respectively (Fig. 1). When required, simvastatin was added together with modified basal diet. The dosage of drug was 10.36 mg/Kg for a total of 14 weeks. The food intake was measured by direct weighing of the food.

Biochemical determinations

At the end of the experiments, the animals were anesthetized through ethereal inhalation, and blood samples were collected through cardiac puncture for measuring the plasma cholesterol, triglycerides, LDL, HDL, Aspartate aminotransferase (AST) activity, urea – blood urea nitrogen (BUN) and creatinine.

The plasma lipid, urea and creatinine measurements were performed in an ADVIA 1650 automated system (Bayer AG, Leverkusen, Germany).

The blood urea nitrogen/creatinine (BUN/Cr) ratio was selected a prior to represent liver damage in animals. This ratio has been cited in other studies and medical textbooks as a marker of liver damage in patients with no renal disease [20, 21].

Liver lipid hydroperoxides

Dosage of lipid hydroperoxides was carried out on methanolic extract of liver tissue as described by Nourooz-Zadeh *et al* [22*]*. A 300 mg portion of the liver right lobe (laparoscopic liver resection) was homogenized in 1 mL methanol, using an electric homogenizer (GGS 27, Bosch). After centrifugation (5000 g, 5 min, 4°C), a 50 µL aliquot of the supernatant (cell-free extract) was stored for further measurement of the total proteins, and 90 µL aliquots were disposed into six centrifuge vials (1,5 mL). To three of these vials, 10 µL of methanolic 10 mM triphenylphosphine was added, thereby generating three blanks. To the other three vials, 10 µL of methanol was added. All the six vials were vortexed and then incubated for 30 min at room temperature. After that, 900 µL FOX 2 (100mM xylenol orange, 4 mM BHT, 25 mM sulfuric acid and 250 mM ammonium ferrous sulfate, 90% methanol, 10% ultrapure water) was added to all vials. After mixing, the samples were incubated for another 30 min at room temperature. The absorbance was measured at 560 nm using a spectrophotometer (Ultrospoc 2000, Pharmacia Biotech). The results were corrected for the extract protein concentration. The method described by Bradford was carried out for this measurement [23].

Peritoneal macrophages activity

Peritoneal macrophages were obtained by centrifugation (200 g for 8 min. at 4°C), washed, and counted using trypan blue solution (1%). Then the peritoneal macrophages were resuspended (10^6 cells/mL) in PBS (pH=7.4) or for culture in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum containing antibiotic solution (10 U/mL streptomycin and 20 U/mL penicillin) [24, 25]. Peritoneal macrophages activity was analyzed using phagocytic capacity,

lysosomal volume and ion production (Superoxide anion, Hydrogen peroxide and Nitric oxide).

Phagocytic capacity

Aliquots of peritoneal macrophages (10^6 cells/mL) were added to the wells of a 96-well flat bottomed tissue culture plate. Then 10 µL of neutral-red stained zymosan (10^8 particles/mL) were added to each well. After incubation for 30 min, the cells were fixed with Banker´s formol-calcium solution (4 % formaldehyde, 2 % sodium chloride, 1 % calcium acetate) for 30 min. The cells were then washed two times and centrifuged at 453 g for 5 min. The neutral-red stain was solubilized by adding 0.1 mL of acidified alcohol (10 % acetic acid, 40 % ethanol in distilled water) to each well. After 30 minutes, the absorbance of each well was read on a plate reader at 550 nm. The results were expressed as absorbance (per 10^6 cells/mL) [24, 25].

Lysosomal volume

The uptake of the cationic dye neutral red, which concentrates in macrophages, was used to assess the volume of the peritoneal macrophages. Twenty microliters of 2% neutral red in PBS was added to 100 µL of peritoneal macrophages per microplate well and incubated for 30 minutes. The cells were then washed twice with PBS by centrifugation (453 g for 5 min.). Neutral red was solubilized by a 30 minutes incubation adding 0.1 mL of 10% acetic acid plus 40% ethanol solution. The absorbance was read at 550 nm, and lysosomal volume was expressed as absorbance (per 10^6 cells/mL) [24–26].

Superoxide anion production ($O_2^{\bullet-}$)

Superoxide anion production was estimated by the nitro blue tetrazolium (NBT – Sigma) reduction assay. Peritoneal macrophages (100 µL) were incubated at 37°C in the absence and presence of 10 µL of phorbol myristyl acetate (final concentration, 4 µM) and 0.2% NBT. After 1 hour the mixture was centrifuged (453 g for 5 minutes), the supernatant was discarded, and the peritoneal macrophages were fixed by adding 100 µL of methanol (50%) for 10 minutes. The plate was centrifuged again, the supernatant was discarded, and the plate was dried. Then 120 µL of KOH (2M) and 140 µL of dimethyl sulfoxide were added to the wells. After 30 minutes the reduction of NBT resulted in the formation of blue formazana. The absorbance was

read at 550 nm. The results were expressed as absorbance (per 10^6 cells/mL) [24–27].

Hydrogen peroxide production (H_2O_2)

Hydrogen peroxide production was based on the horseradish peroxidase-dependent conversion of phenol red into a colored compound by H_2O_2 [27]. Peritoneal macrophages (100 µL) were incubated in the presence of glucose (5 mM), phenol red solution (0.56 mM), and horseradish peroxidase (8.5 U/mL) in the dark for 1 hour at 20°C. The absorbance was read at 620 nm. The results were expressed as absorbance (per 10^6 cells/mL).

Nitric oxide production (NO)

Nitric oxide production was measured as nitrite (NO_2^-) by the Griess reaction [28]. Aliquots of peritoneal macrophages (100 µL) were incubated for 2 hours at 37 °C into wells of a 96-well flat-bottomed tissue culture plates. The plates were washed twice with PBS to remove the nonadherent cells. The remaining cells were incubated for 24 hours in the absence or presence of lipopolysaccharide (10 µg/mL). Equal volumes of cell culture supernatant and Griess reagent were incubated for 10 minutes at room temperature, and the absorbance was read at 550 nm and expressed as absorbance (per 10^6 cells/mL).

Statistical analysis

The data are presented as mean ± SEM values. Statistical analysis was performed by a two-tailed unpaired Student´s *t* test (GraphPad [San Diego, CA, USA] Prism version 5 software) and it was carried out at the 5 % significance level.

RESULTS

In this study it was studied hypolipidemic and antiatherosclerotic potential of *P. ostreatus* cultived by submerged fermentation in HFD fed rats as indicated in Fig.3 1. During the experiment period the animals did not show any visible signs of discomfort and all the groups consumed the same amount of feed (data not shown).

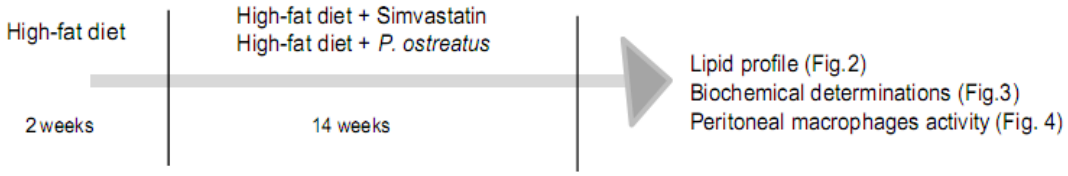

Figure 3 1 - Study design. Hypolipidemic potential and antiatherosclerotic potential of *P. ostreatus* cultived by submerged fermentation in HFD fed rats.

Biochemical determinations – Hypolipidemic effect

Levels of lipid parameters were calculated using enzymatic-colorimetric method and, as expected, HFD animals showed an increase in plasma cholesterol and LDL. However, the triglycerides and HDL levels exhibited no changes compared with control animals. Lipid parameters (plasma cholesterol, triglycerides, LDL and HDL) are in Fig. 3 2.

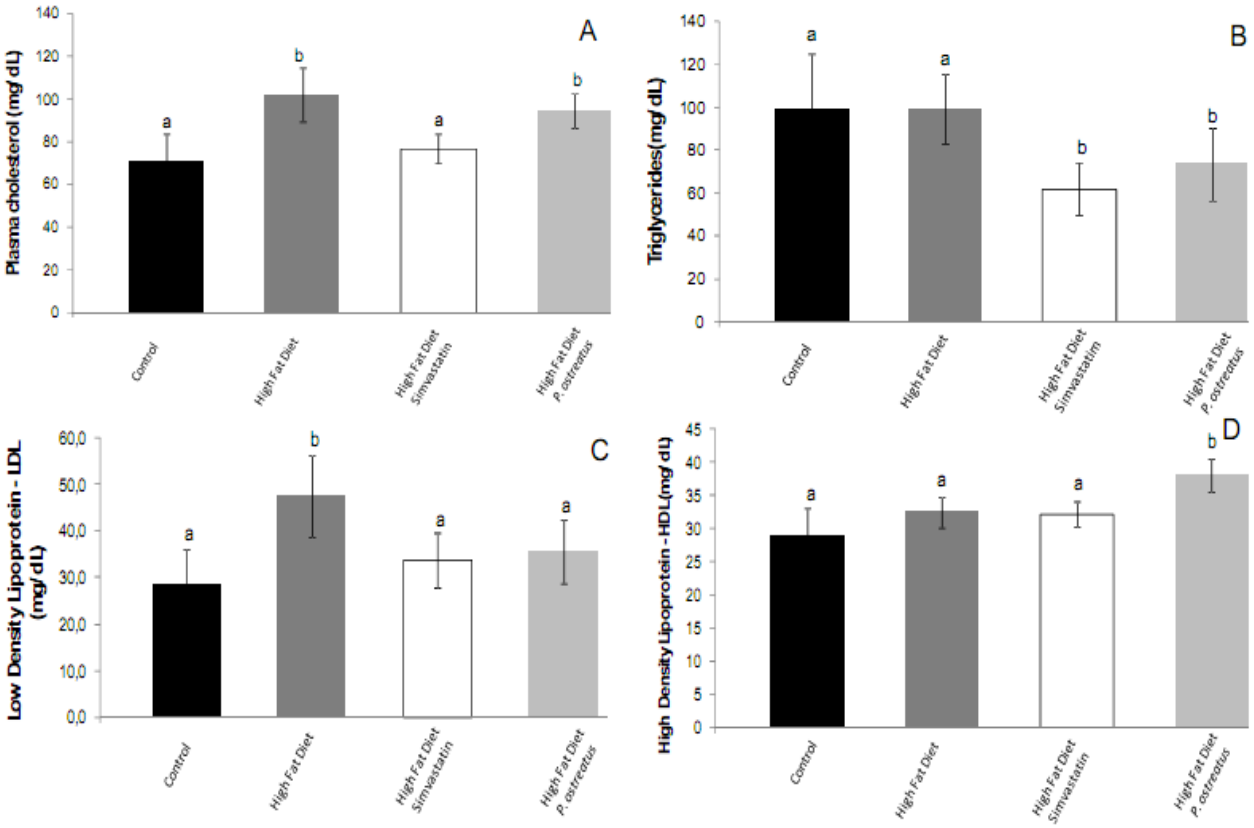

Figure 3 2 - Lipid profile. Plasma cholesterol, triglycerides, LDL and HDL in rats fed HFD and HFD supplemented with simvastatin and biomass (*P. ostreatus).* Data are mean ± SEM values of ten rats per treatment group. (A,B,C,D) ^{b} p<.05 compared to control group.

Triglycerides and LDL showed a trend towards a decrease in values over supplementation with *P. ostreatus* and an increase in HDL levels. The mean ± SEM of triglycerides and LDL in the presence of HFD models and *P. ostreatus* was 99 ± 16.35/ 73.4 ± 16.9 and 47.6 ± 8.75/ 35.6 ± 6.88 respectively. Positive response was observed by simvastatin such as plasma cholesterol, triglycerides and LDL levels in animals models. Interestingly, synthetic hypolipidemic (simvastatin) and *P. ostreatus* showed similar trends (triglycerides and LDL levels), being 61.75 ± 12.18/ 73.4 ± 16.9 and 33.8 ± 5.91/35.6 ± 6.88 respectively.

Hepatoprotective effect

Additional experiments were performed to determine the liver damage in rats fed HFD (Fig. 3 3). Serum urea and BUN/Cr ratio were significantly decreased in HFD presence and, as illustrated in Fig. 3[c, d], HFD increased AST and lipid peroxidation levels. We demonstrated that the mean values of all parameters (Fig. 3 3 [a, b, c, d]) were affected by HFD. Conversely, AST and lipid peroxidation showed liver recovery in simvastatin and *P. ostreatus* groups. Urea and lipid peroxidation levels were evaluated using urease/NADH and ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine [22].

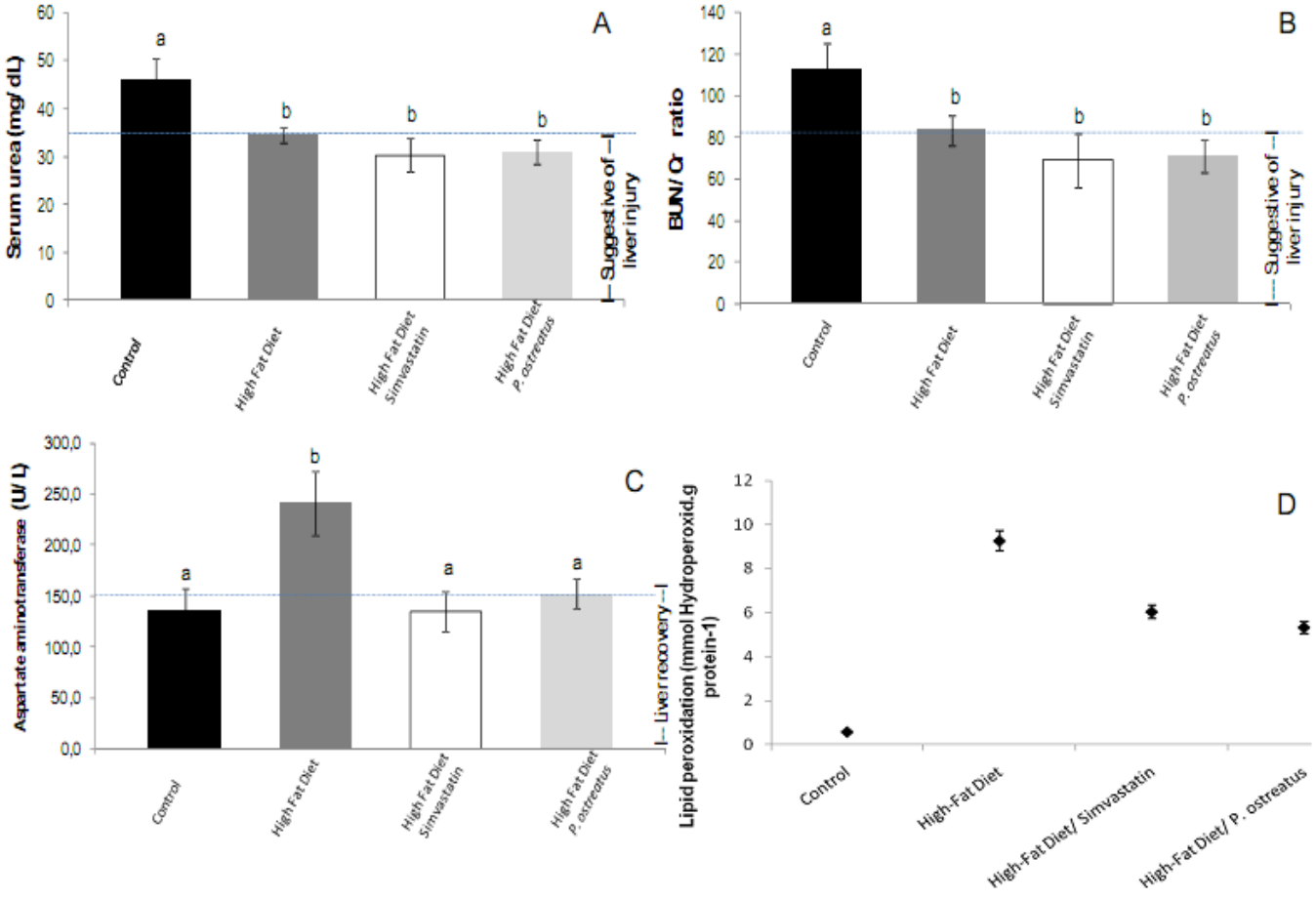

Figure 3 3 - Biochemical determinations. (A, B, C) Serum urea, BUN/creatinine ratio, AST activity in rats fed HFD and HFD supplemented with simvastatin and biomass (*P. ostreatus).* (D) Lipid peroxidation in rat liver homogenate. Data are mean ± SEM values of ten rats per treatment group. [b] $p<.05$ compared to control group.

Peritoneal macrophages activity - atherosclerotic activity

Peritoneal macrophages were assessed by metabolic activity (Hydrogen peroxide, superoxide anion and nitric oxide production) and morphological indicators as lysosomal volume and phagocytic capacity (Fig.4). HFD did not induce any activity in peritoneal macrophages. Interestingly, simvastatin and *P. ostreatus* decreased metabolic activity as related to the hydrogen peroxide and superoxide anion levels implicated (Fig. 3 4 [a, b]), being approximately 75.1 and 66.9 % of inhibition.

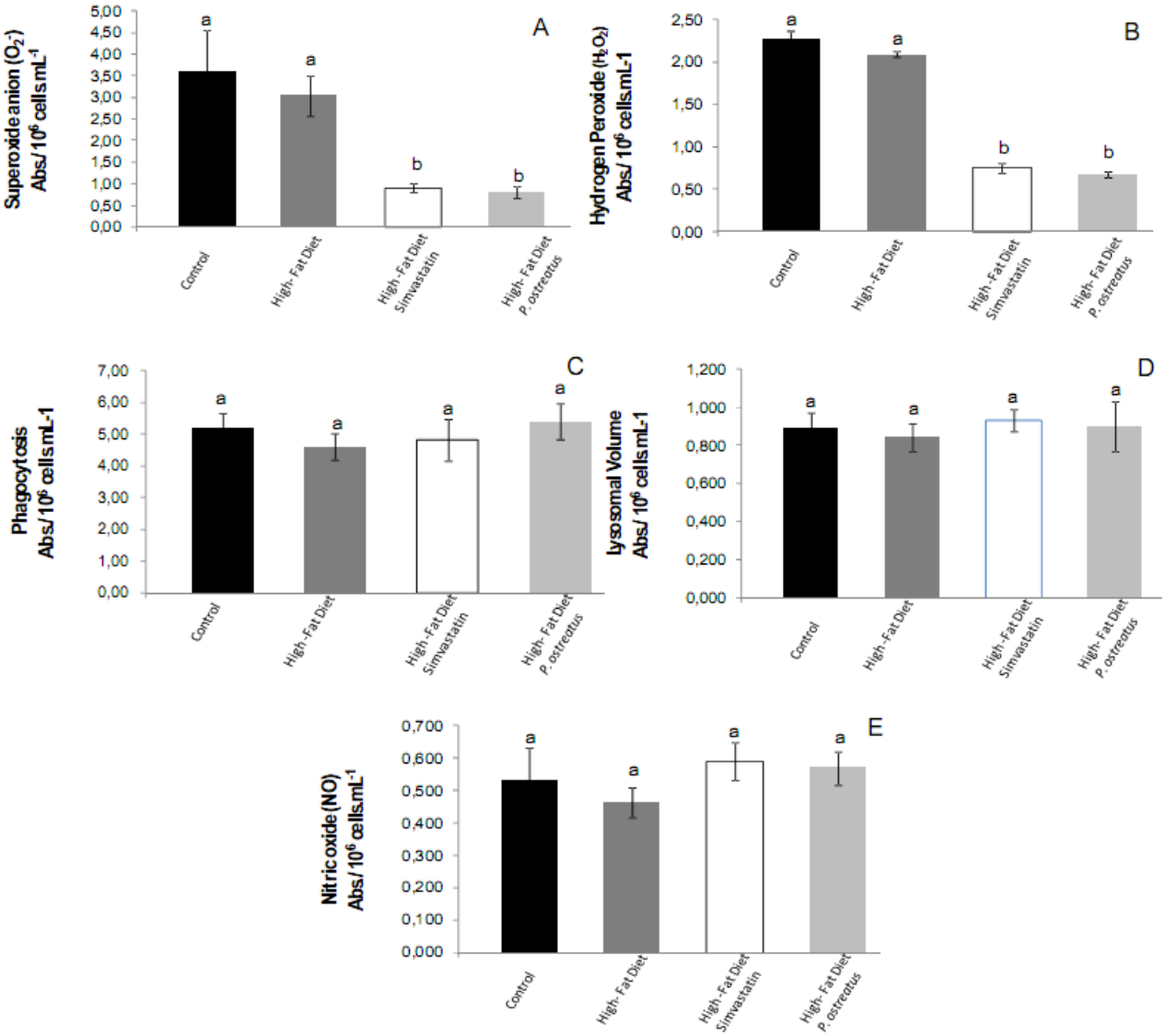

Figure 3 4 - Peritoneal macrophages activity. (A, B, E) Superoxide anion, Hydrogen peroxide and nitric oxide production, (C, D) phagocytic capacity and lysosomnal volume by peritoneal macrophages from rats treated with HFD and HFD

supplemented with simvastatin or *P. ostreatus*. Data are mean ± SEM values of ten rats per treatment group.[b] p>.05 compared to control group.

DISCUSSION

The main objective of this study was to evaluate hypolipidemic and antiatherosclerotic potential of *P. ostreatus* cultived by submerged fermentation in HFD fed rats in order to contribute to a better understanding of its application in the treatment of hyperlipidemia and atherosclerotic lesions. The basic difference between the previous studies and our work is that we extend these data in *P. ostreatus* cultived by submerged fermentation. Submerged culture have been considered as having a successul outcome in respect to high-quality biomass production [13, 14]. In addition, Sandhya et al. (2005) has reported neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation and found a 3.5-fold increase in enzyme yield in solid-state fermentation when compared to submerged culture [15, 16]. This could be explained by the fact that physical properties, e.g. pH, aqueous activity, in each fermentation process have influencied levels of bioactive compouds and biological activities. Thus, comparative studies between solid-state fermentation and submerged fermentation can show different levels of bioactive compouds and biological activities.

Will *P. ostreatus* cultived by submerged fermentation show hypolipidemic effects as well as those effects obtained by solid-state fermentation? Our study goes a fair way to providing as answer that *P. ostreatus* obtained from submerged fermentation is a potential candidate to complement currently available drug regiments for hyperlipidemia. The results showed that, as expected, HFD produced significant increases in total cholesterol and LDL in our animals who had been on a long-term intake regimen. Synthetic hypolipidemic (simvastatin) and *P. ostreatus* showed similar trends to cause decreased triglycerides and LDL levels. In light of the recent studies, it is possible that compounds (lovastatin, glucans, steroids, niacin, CETP inhibitors) derived from the cell body *P. ostreatus* had hypolipidemic activity [29–31]. For example, clear and final conclusions can be drawn regarding the action mechanisms of glucans – the newest hypolipidemic agent. The observed decrease in cholesterol level may be due to an increase in bile acid excretion induced by glucans [31]. Although the decrease in cholesterol levels was not noted in our study, it was

observed decrease in LDL and triglycerides levels which were consistent with those of previous studies which used *P. ostreatus* obtained from solid-state fermentation (fruiting bodies). At least two reasons can be devised for invariable levels of cholesterol in our study: (a) comparative studies between solid-state fermentation and submerged fermentation can show different levels of bioactive compouds and biological activities obtained in these thechniques; (b) we have used high fat content (20%) in our diet preparation which is unlike other studies [6,7; 13-16].

Additionally, all statins have been observed to cause myopathy, and the risk of adverse effects on muscle increases with the use of high doses [32]. Therefore, *P. ostreatus* could decreases the use of high doses and adverse effects and, thus, be really an alternative treatment option. *P. ostreatus* also have a positive response with regard to HDL levels which is able to remove excess cell cholesterol and protect against atherosclerosis [33].

Liver damages have been observed after 16 weeks supplementation period in HFD groups (Fig. 3 3). Despite that treated groups (simvastatin and *P. ostreaus*) in serum urea and BUN/Cr ratio (Fig. 3 3 [a, b]) are not able to normalize urea cycle enzymes, it was able to restore AST activity and decrease oxidative damage (lipid peroxidation), clearly demonstrating a liver recovery. The assessment of oxidative damage by the lipid peroxidation in tissue exposed to oxidative stress has been proposed to evaluate tissue damage [34, 35]. As a matter of fact, correlation between HFD and improved oxidative stress was reported and observed in our study [34–36].

Atherosclerosis is a disease involving endothelial dysfunction, oxidative stress, immunity, inflammation and thrombosis. The pathogenesis of atherosclerotic lesions is characterized by systemic, chronic and progressive inflammation disease which has a known effect on build-up of lipid-rich plaques within the walls of large arteries [10]. Moreover, oxidative stress in macrophages has been documented as important cause of development of atherosclerosis [9, 17–19]. Notably, a high level of circulating lipid in the blood is one of the risk factors for the development of coronary atherosclerotic lesions [8–11]. For this reason, it is interesting to note that both hypolipidemic and antiatherosclerotic effects should be found in potential drugs for hyperlipidemia. The findings in this study confirm that *P. ostreatus* biomass and simvastatin in our experimental conditions are sufficient to decrease oxidative stress (antiatherosclerotic effects) and lipid parameters. Decreased effects of statin on the oxidative stress by monocytes were also reported [9].

CONCLUSIONS

The results of this study indicate that *P. ostreatus* can produce beneficial effect on lipid profile and macrophages activity during a HFD for a long-term intake. In this condition, *P. ostreatus* drecreased LDL and triglycerides and exert positive effects on HDL. *P. ostreatus* caused greater inhibitory effects on oxidative stress and it promoted liver protection against HFD. This possibility is in accordance with the requirements to suggest new potential hypolipidemic and antiatherogenic agents. Probably, *P. ostreatus* supplementation cannot replace the use of currently available drug regiments for lipid reduction, but can complement them. They may also enable the use of lower doses of therapeutic drugs, thereby decreasing the risk of dose-related side effects.

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CHAPTER IV

EFFECTS OF *Cordyceps sinensis* ON MACROPHAGE FUNCTION IN HIGH-FAT DIET FED RATS AND ITS ANTI-PROLIFERATIVE EFFECTS ON IMR-32 HUMAN NEUROBLASTOMA CELLS

EFFECTS OF *Cordyceps sinensis* ON MACROPHAGE FUNCTION IN HIGH-FAT DIET FED RATS AND ITS ANTI-PROLIFERATIVE EFFECTS ON IMR-32 HUMAN NEUROBLASTOMA CELLS

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ABSTRACT

Macrophages have been considered an elusive yet emerging therapeutic target in tumor development since they are an important component in tumor microenvironment. The purpose of the present study was to evaluate the effect of *C. sinensis* on macrophage function (a component of tumor microenvironment which can alter the virulence of cancer) in high-fat diet fed rats - an aggravating factor of macrophage polarization. IMR-32 human neuroblastoma cell cytotoxicity was also investigated. The following parameters were observed to evaluate macrophage function: superoxide anion, hydrogen peroxide, nitric oxide, lysosomal volume and phagocytic capacity. High fat diet (HFD) plus *C. sinensis* supplementation promoted a decreased superoxide anion and hydrogen peroxide levels as well as lysosomal volume and phagocytic capacity. Nitric oxide was increased in the same group. In

summary, *C. sinensis* offered an important anti-tumoral perspective from the standpoint of the tumor microenvironment and *in vitro* IMR-32 cytotoxicity.

Keywords: *Cordyceps sinensis*, macrophage funcion, high-fat diet, anti-tumoral

INTRODUCTION

Currently, medical mushrooms are becoming increasingly popular as foods and supplements with special health properties. Of all the medicinal mushrooms, *C. sinensis*, an ascomycete, is officially recognized as a Chinese medicinal treasure [1]. Recently, previous studies suggest that the *C. sinensis* strongly inhibits T cells activity and reduce interferon gama (IFN-γ) production [2]. Notoriously, IFNs play a central role in the modulation of immune responses and macrophage activation. Furthermore, previous studies demonstrated that aqueous extract of *C. sinensis* significantly inhibited the activity of macrophage phagocytosis assessed by colloidal carbon clearance assay as well as the *C. sinensis* increased nitric oxide concentration. [3,4]. Thus, we are mainly interested in the effects of *C. sinensis* on other aspects of macrophage activation such as oxidative burst as well as the activity of macrophage phagocytosis by other method such as zymosan phagocytosis assay.

Macrophages are well known to play an important role in innate immune system as initiators and effectors [5]. However, macrophages also markedly increase the virulence and progression of cancer because activated macrophages can enhance tumor cell invasion, migration and angiogenesis which are mainly involved in the tumerogenesis process [6–9]. Thereby, current interest in the effect of potential agents such as *C. sinensis* on modulation of the macrophage function is centered on the possibility that the *C. sinensis* could have a beneficial effect on virulence and progression of cancer through a reduction of macrophage activation.

Evaluation of macrophage function has been linked to certain parameters such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and nitric oxide (NO) production as well as lysosomal volume and phagocytic capacity [10,11]. Only in the past decade the importance of these oxidative burst parameters (H_2O_2, O_2^- and NO) and phagocytic capacity became more and more apparent in the occurrence and progression of cancer [6,12,13].

Similarly, HFD is the most prominent cause of hyperlipidemia for both men and women in most developed countries and it is considered to put an individual at greater risk of hypertension, heart disturbances, diabetes and obesity. In addition, HFD is also associated with an increased contribution to virulence and incidence of cancer because there is clear correlation between body fat and elevated tumor growth hormones or HFD and higher tumor volume [14,15]. Most important, studies have been demonstrated that HFD can promotes an altered macrophage polarization which could reflect on an altered virulence of cancer [9,16]. Thus, add an aggravating factor such as HFD which is correlated with altered macrophage polarization can be interesting to evaluate the oxidative burst and morphological parameters of macrophages in hyperlipidemic animals and its speculations for anti-cancer potential agents [16–18].

Neuroblastoma is a neoplasm of the sympathetic nervous system. This disease is the second most common extracranial malignant tumor of childhood and the most common solid tumor of infancy. Surgical resection, chemotherapy, or radiotherapy procedures are indicated upon a patient´s risk stratification. Survival rates for patients who have International Neuroblastoma Staging System (INSS) stage 1 are excellent with surgery alone. The introduction of new agents, when INSS > 1, is essential to reduce the use of chemotherapy and radiotherapy [19].

In this study the main focus will address the outcome of *C. sinensis* on anti-tumor perspectives through the evaluation of macrophage function (a component of tumor microenvironment which could alter the virulence of cancer) in hyperlipidemic rats – an aggravating factor of altered macrophage function (Fig. 4 1). Finally, cytotoxicity test against IMR-32 human neuroblastoma cells after treatment with water extract of *Cordyceps sinensis* were evaluated.

MATERIAL AND METHODS

Fungal strain

Cordyceps sinensis PPGEBB was obtained from the *Banco de Cepas do Departamento de Engenharia de Bioprocessos e Biotecnologia*, UFPR (Curitiba, Brazil). The strain was maintained on nutrient agar slants.

Submerged culture conditions

Cultures were carried out in 1 L Erlenmeyer flasks, with 400 mL of a basal medium containing per liter: dextrose 30 g, peptone 8 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g at 25°C shaken at 120 rpm, pH was adjusted to 5.5. For experimental diets, mycelium was then removed by filtration on filter paper [1].

Experimental diets

HFD was prepared using a laboratory animal feed (Labina, Purina®, São Paulo, Brazil) with the following ingredients (g/100g): lard, 14 and hydrogenated vegetable fat, 6. To prepare it, the pulverized standard diet and melted lipids (lard and hydrogenated vegetable fat) were mixed. Control group was fed with basal diet without modification. When required, *C. sinensis* biomass was added together HFD. The dosage of biomass was 20% (w/w) (biomass/feed) for a total of 16 weeks [20].

Animals

All procedures involving animals were approved by the Positivo University Committee for Animal Welfare. Thirty male Wistar rats, 30 days weighing 110 g (10 ± g) were divided into three groups (ten per group). The animals were kept in the animal house at a temperature of 24 ±2 °C with a 12/12 hour light/dark cycle for 4 months and fed with the respective diets and water *ad libitum.*

Peritoneal macrophages activity

Peritoneal macrophages were obtained by peritoneal cavity washing (5mL of PBS) followed by centrifugation (200 g for 8 min. at 4°C). Then the macrophages were counted using trypan blue solution (1%) and were resuspended (10^6 cells/mL) in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum containing antibiotic solution (10 U/mL streptomycin and 20 U/mL penicillin). Peritoneal macrophages activity was evaluated using superoxide anion, hydrogen peroxide and nitric oxide production, as well as phagocytic capacity and lysosomal volume [10,21]. Chemicals and cell culture medium used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Superoxide anion production ($O_2^{\bullet-}$)

Superoxide anion production was evaluated by the reduction of nitroblue tetrazolium. Peritoneal macrophages (10^5 cells in 0.45 mL of PBS) were incubated for 1h at 37°C in the presence of 0.03 mL of phorbol myristyl acetate (5 μM) and 0.1% (wt/v) nitroblue tetrazolium. Then the mixture was centrifuged (453 g for 5 minutes), the supernatant was discarded, and the peritoneal macrophages were fixed

by adding 100 µL of methanol (50%) for 10 minutes. The plate was dried and 120 µL of KOH (2M) and 140 µL of dimethyl sulfoxide were added to the wells. After 30 minutes the reduction of NBT resulted in the formation of blue formazan. The absorbance was read at 550 nm [21,22].

Hydrogen peroxide production (H_2O_2)

H_2O_2 production was evaluated using an assay based on the horseradish peroxidase-dependent conversion of phenol red into a colored compound by H_2O_2. Peritoneal macrophages (100 µL) containing 10^5 cells were incubated in the presence of glucose (5 mM), phenol red solution (0.56 mM) and horseradish peroxidase (8.5 U/mL) in the dark for 1 hour at 20°C. H_2O_2 production was detected spectrophotometrically at 620 nm [21,23].

Lysosomal volume

The lysosomal volume of the peritoneal macrophages was assessed by the uptake of the cationic dye neutral red which concentrates in macrophage lysosomal system. 20 µL of 3% neutral red in PBS were added to 0.1 mL of peritoneal macrophages suspension per plate well during 30 min. The cells were then washed doubly and centrifuged at 453 g for 5 min. The neutral red stain was solubilized by adding 0.1 mL of 10% acetic acid plus 40% ethanol solution. The absorbance was read at 550 nm and lysosomal volume was showed as absorbance [10,21].

Phagocytic capacity

0.1 mL of peritoneal macrophages suspension containing 10^5 cells were added to the wells of a 96-well flat bottomed tissue culture plate. Then 10 µL of neutral-red stained zymosan (10^8 particles/mL) were added to each well. The plate was incubated for 30 min. After incubation time, the cells were fixed with Baker´s

formol-calcium solution (4% formaldehyde, 2% sodium chloride and 1% calcium solution) for 30 min. The wells were then washed doubly and centrifuged at 453 g for 5 min. 0.1 mL of acidified alcohol (10% acetic acid, 40% ethanol and distilled water q.s) was utilized to solubilize neutral-red stain. After 30 min, the absorbance of each well was read on a plate reader at 550 nm [21,24].

Nitric oxide production (NO)

NO production was evaluated as nitrite (NO_2^-) by the Griess reaction. Macrophages (100 µL) were incubated for 2 h at 37°C in a 96-well flat-bottomed tissue culture plate. Then the plate was washed twice with PBS to remove the nonadherent cells. The remaining cells were incubated for 24 h in the presence and absence of lipopolysaccharide (10 µg/mL). 100 µL of Griess reaction were added for 10 minutes at room temperature and the absorbance was measured at 550 nm [21,25].

Cytotoxicity assay

The IMR-32 human neuroblastoma and fibroblasts cells were obtained from the cell bank of the Pelé Pequeno Príncipe Institute. The cells were cultived in 25 cm^2 culture flasks in Dubelcco´s Modified Eagle Medium (DMEM; Gibco). The medium was supplemented with 5% fetal bovine serum (Gibco). The cells were grown in a CO_2 (5%) incubator that was humidified at 37°C. The MTT (Thiazolyl Blue Tetrazolium Bromide) assay was performed according to the method described by Mosmann [26]. IMR-32 and fibroblasts cells were seeded in a 96-well culture plate (10^5 and 3.10^3 cells/well respectively) and incubated for a period of 24 h to stabilize. Next, the cells were then treated with various dilutions (1/8, 1/4, 1/3, 1/2) from hot water extract (F) of *Cordyceps sinensis* (40g dry biomass powder was mixed with 600 mL distilled water and heated at 90°C in a water bath for 4h, followed by centrifugation (453 g for 5 min). Next , the treatment medium was discarded, and the plate was incubated at 37°C for 4 h with MTT diluted in phosphate buffered saline. The MTT was then discarded, and dimethyl sulfoxide (DMSO) was added to dissolve

the formazan. The optical density was measured at 550 nm using a spectrophotometer plate reader.

Treatment of data

The statistical significance of the differences between parameters obtained in the experiments was assessed by student´s test. Treatment of data was carried out at the 5% significance level. Data are expressed as means ± SEM.

RESULTS

The effect of *C. sinensis* on macrophage function was made by observing certain parameters such as hydrogen peroxide, superoxide anion and nitric oxide production as well as lysosomal volume and phagocytic capacity. Reaction mechanisms are referenced in Section Material and Methods.

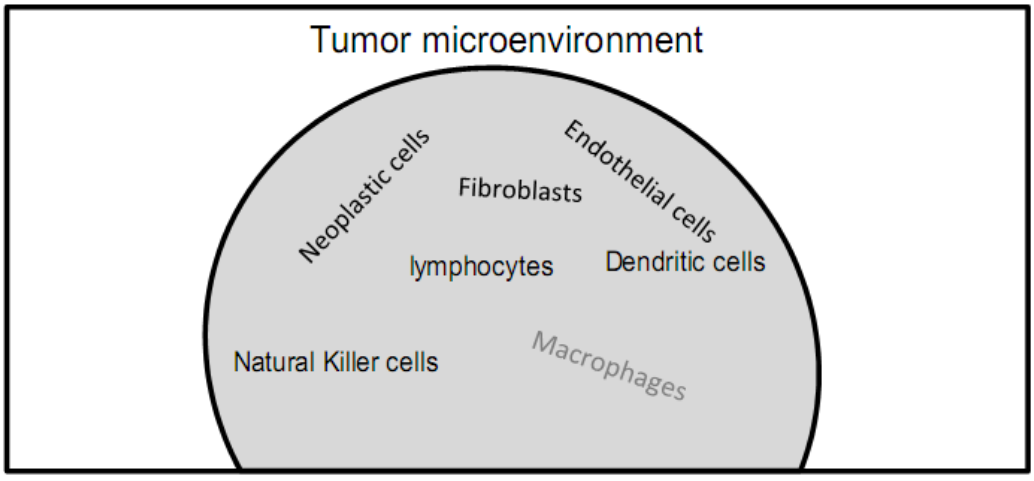

Figure 4 1 - Tumor microenvironment is composed of proliferating neoplastic cells, extra cellular matrix produced by fibroblasts, a vascular network of endothelial cells and cellular components of immune system such as macrophages

Oxidative burst – superoxide anion, hydrogen peroxide and nitric oxide

For evaluation of oxidative burst, superoxide anion, hydrogen peroxide and nitric oxide were observed. The results are shown in Fig. 4 2, 4 3 and 4 4. We have

found that, at the end of the experiments (4 months), *C. sinensis* decreased superoxide anion (42.5%) and hydrogen peroxide (33.33%) (Fig. 4 2 and 4 3). However, according to the figure 4 4, *C. sinensis* increased nitric oxide levels (32.07%). Interestingly, HFD group did not show effects on superoxide anion, hydrogen peroxide and nitric oxide levels.

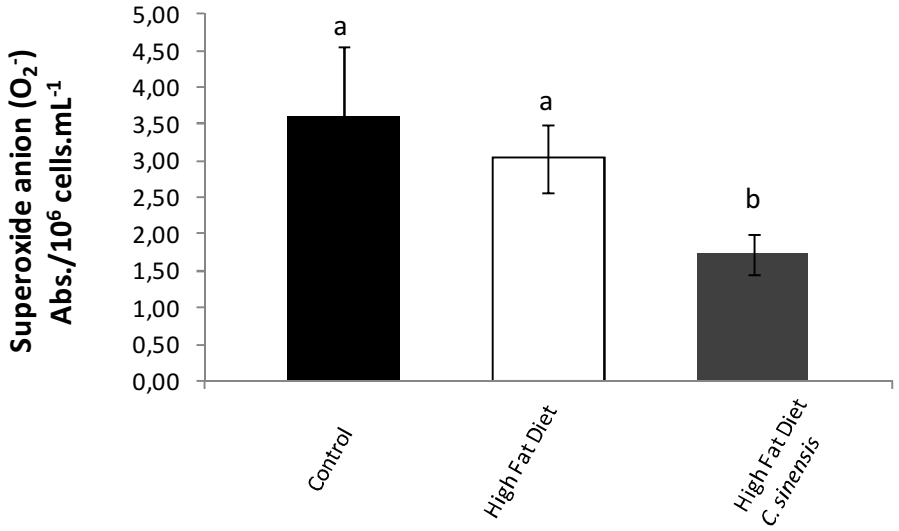

Figure 4 2 - Effects of *C. sinensis* on measured superoxide anion by nitro blue tetrazolium reduction assay. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control

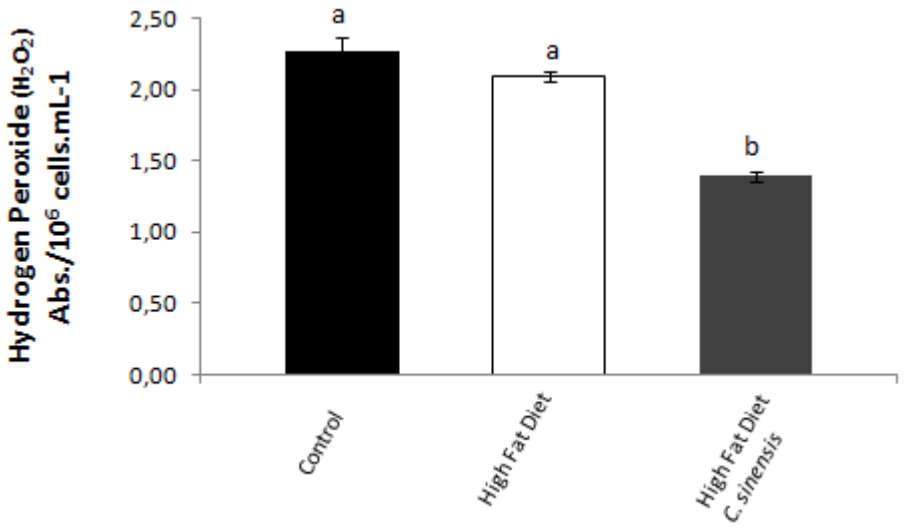

Figure 4 3 - Effects of *C. sinensis* on measured hydrogen peroxide by horseradish peroxidase. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control

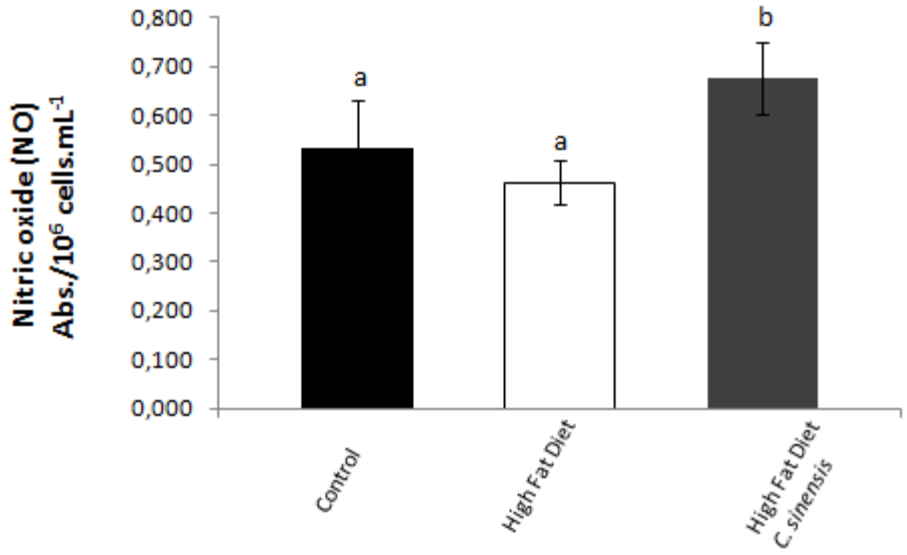

Figure 4 4 - Effects of *C. sinensis* on measured nitric oxide by Griess reaction. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control

Morphological parameters – lysosomal volume and phagocytic capacity

Fig. 4 5 and 4 6 shows that oxidative burst changes were accompanied by a decrease in morphological parameters as can be seen in lysosomal volume and phagocytic capacity. Thereby, *C. sinensis* showed inhibitory effects on oxidative burst

and morphological parameters. Once again, HFD did not alter lysosomal volume and phagocytic capacity.

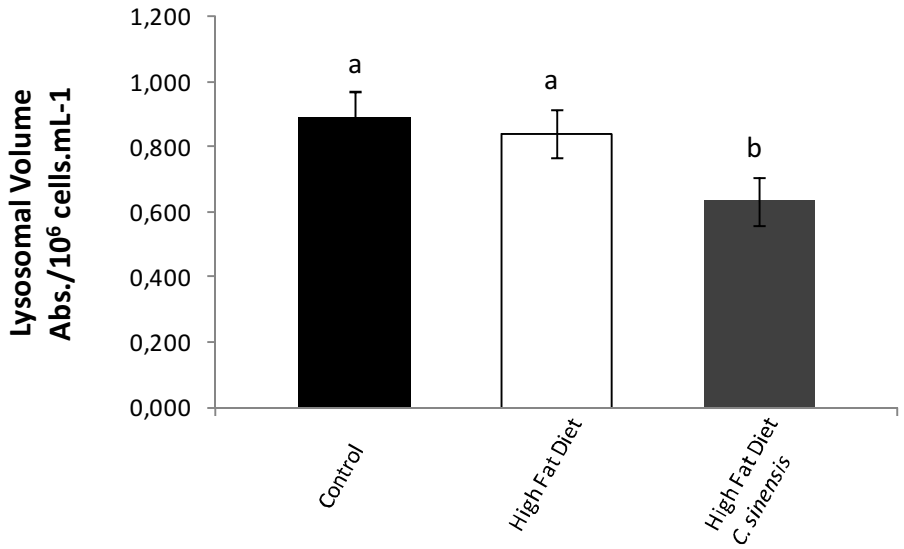

Figure 4 5 - Effects of *C. sinensis* on measured lysosomal volume by uptake of the cationic dye neutral red. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control

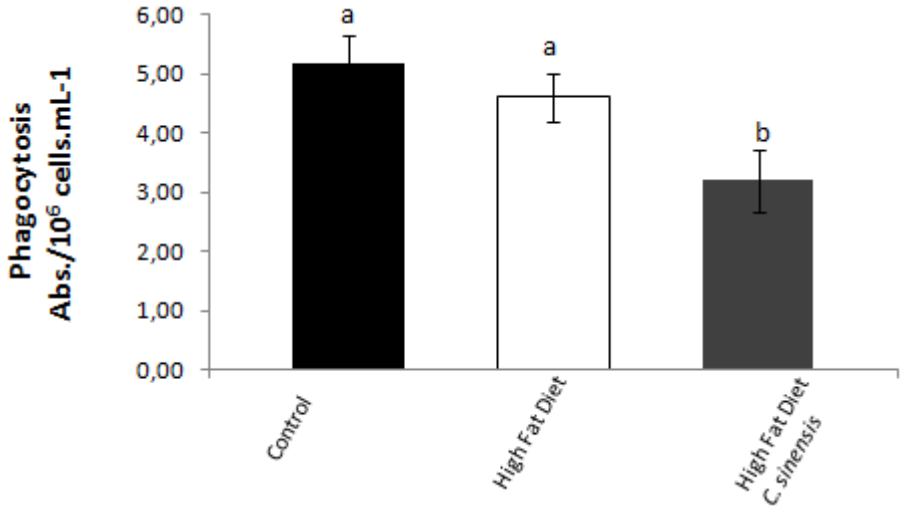

Figure 4 6 - Effects of *C. sinensis* on measured phagocytosis by zymosan assay. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control

Antitumor activity

Fig. 4 7 and 4 8 shows the cell response for the treatment effects from water extract of *Cordyceps sinensis* against IMR-32 and fibloblasts cells. Water extract of *Cordyceps sinensis* exhibited moderate antitumor activity with about 20% inhibition of the IMR- 32 cell growth. Compared to the fibloblasts cytotoxicity assay, the water extract of *Cordyceps sinensis* is evidently non-toxic.

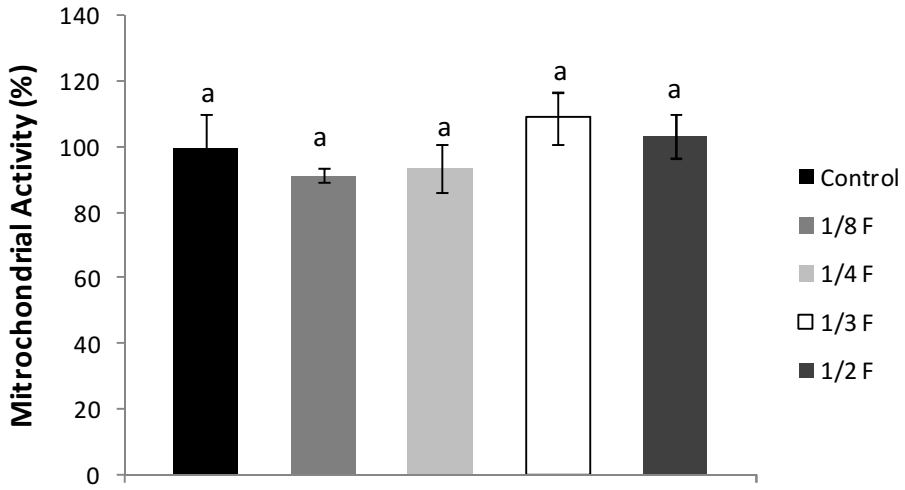

Figure 4 7 - Effects of water extract of *C. sinensis* on measured fibloblasts activity by MTT. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control

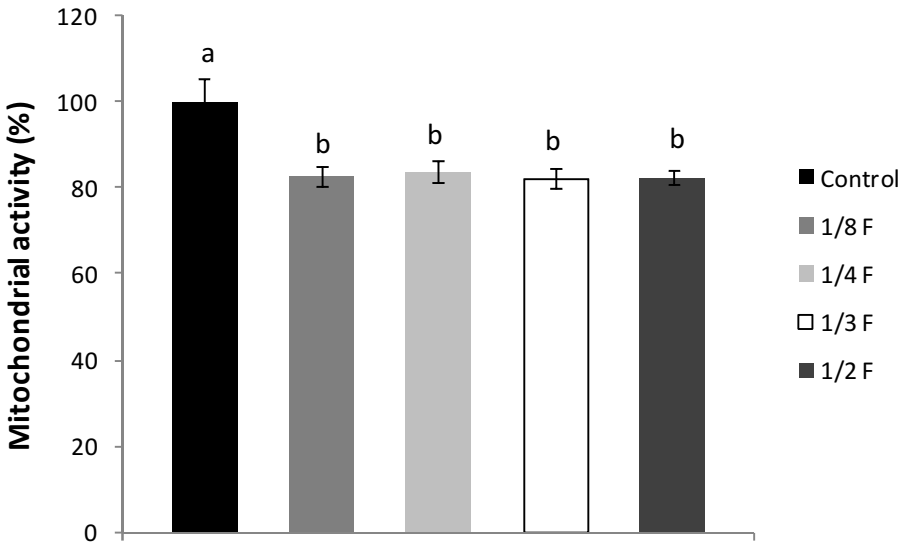

Figure 4 8 - Effects of water extract of *C. sinensis* on measured IMR-32 neuroblastoma cell activity by MTT. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control

DISCUSSION

The main objective of the present study was to evaluate the influence of *C. sinensis* on the macrophage functions by peritoneal macrophages. In spite of the well known effect of *C. sinensis* on macrophage phagocytosis assessed by colloidal carbon clearance assay and nitric oxide concentration, here we extend these data in macrophage oxidative burst and macrophage phagocytosis capacity by zymosan phagocytosis assay – this assay provides substantial assistance as an additional parameter in the evaluation of phagocytosis. Although no tumor has yet been induced in our current experimental design, macrophages can be a new target since they can appears in tumor microenvironment. New treatments that have additional targets are interesting therapeutic approaches [27]. In addition, there is no information about IMR-32 cells when subjected to water extract from *Cordyceps sinensis.* Up till now, *C. militaris*, *C. takaomontana* and *C. sphecocephala* were evaluated against Neuro 2A and SK-N-SH cells [28–30].

The hypothesis of this study was that *C. sinensis* would alter macrophage function for which there a strong correlation with anti-tumoral potential. For a long time macrophages has been known as a component of innate immune system [5]. However, previous study have been demonstrated that macrophages also markedly increase the virulence and progression of cancer because activated macrophages can enhance tumor cell invasion, migration and angiogenesis which are mainly involved in the tumerogenesis process [6–9]. Thus, the reduction of macrophage activation could serve as alternative therapy to complement currently available drug regiments for tumerogenesis since their prominent role in tumor initiation, development and metastasis [6–9,31].

The data collectively demonstrate that *C. sinensis* decreased macrophage function as observed by lower levels of superoxide anion, hydrogen peroxide, lysosomal volume and phagocytic capacity (zymosan phagocytosis assay). Lower levels of phagocytic capacity by colloidal carbon clearance assay are also implicated in previous studies about effect of *C. sinensis* on macrophage function [4]. Overall, *C. sinensis* was able to reduce oxidative burst and morphological parameters. These striking observations may change positively the tumor microenvironment. Nevertheless, controlled-delivery systems for targeting tumor using this alternative therapy are necessary to release the agents exactly where they should act to avoid systemic distribution and adverse effects [32,33].

The close correlation between low levels of superoxide anion and hydrogen peroxide provided the evidence supporting that the inhibition mechanism occurs at level of enzymes that catalyze the superoxide anion production which will reflect in lower hydrogen peroxide prodution since the hydrogen peroxide is a end product from superoxide anion metabolism [34,35].

Nitric oxide is key mediator involved in many pathological and physiological processes. Our findings show that there was an increase in nitric oxide levels in group treated with HFD and *C. sinensis*. Previous studies also found an increase in NO production by *C. sinensis* [3]. Despite higher levels of nitric oxide, superoxide anions were decreased in same group – the toxicity of nitric oxide is linked to its ability to combine with superoxide anions to form peroxynitrite which is an oxidizing free radical that can cause DNA fragmentation [36]. High levels of peroxynitrite, a metabolic derivative from nitric oxide, can modify functional proteins leading to tumor development [36,37]. Moreover, increased levels of nitric oxide has been revealed interesting features such as participation in anti-tumor mechanisms of potential agents [38,39]. Thus, nitric oxide promotes an antitumorogenic environment.

In the experiments changes observed in IMR-32 neuroblastoma cell. Water extract of *Cordyceps sinensis* exhibited moderate antitumor activity with about 20% inhibition of the carcinogenic cell growth. Other fungal genres such as *C. militaris*, *C. takaomontana* and *C. sphecocephala* have already been demonstrated to have effectiveness in lowering neuroblastoma cell activity (Neuro 2A and SK-N-SH cell). Therefore, these studies previously reported that *Cordyceps spp*. may exert their antitumor activity against neuroblastoma cell through the mechanism of inducing apoptosis [28–30].

The understanding of HFD effect on macrophage function is very limited. Previous study underscores a distinct macrophage polarization upon short term high fat diet feeding [16]. Classical (M1) and alternative macrophage polarization (M2) induce pro-inflammatory and anti-inflammatory cytokines respectively. M1 macrophages play a high activity against microorganisms while studies suggest an important role of M2 macrophages in tumor progression [40]. M1 form may also be

present in cancer and has been associated with its survival time [41]. The observation about an altered macrophage polarization upon short term high fat diet feeding leaves room to the intriguing hypothesis that HFD could also exert some effect on oxidative burst in tumor-associated macrophages. Reactive oxygen species which can be produced by oxidative burst/oxidative stress in tumor associated macrophages has become widely viewed as an underlying condition in cancer and tumors [12,42,43]. HFD is believed to promote pro-inflammatory responses such as macrophage activation in adipose tissue, which contributes significantly to obesity-associated complications [16]. Contrary to our expectations, the results showed that HFD did not affect the macrophage parameters. In our experimental design, we did not observe alteration in body weight (data not shown) although the lipid profile has changed [20]. This may explain why we did not visualize in HFD group alteration in macrophage parameters since macrophage activation has been linked to body fat [44,45]. This clearly suggests that HFD as an aggravating factor to evaluate anti-tumoral potential through macrophage function in experimental design should be useful as long as the body fat increase.

CONCLUSIONS

The main finding from the results presented here is that *C.sinensis* displayed strong effect on macrophage function, particularly for superoxide anion, hydrogen peroxide, lysosomal volume and phagocytic capacity which were decreased and nitric oxide which was increased. Thus, our data demonstrate a decrease in macrophage function although nitric oxide has increased. Interestingly, macrophages have been associated with tumor development and, apparently, new therapies which are specially directed against tumor-associated macrophages could offer benefits for treatment of tumor. A high level of nitric oxide was considered important since it show

interesting features such as participation in anti-tumor mechanisms. *C.sinensis* not only decreased macrophage function, but also IMR-32 neuroblastoma cell activity. Unfortunately, we did not observe any affect of HFD as we had hypothesized at the beginning of our study. We do not intend to replace the use of currently available drug regiments for treatment of tumor, but complement them through integration of substances which have effect on macrophage function - a component of tumor microenvironment. There are interest in further investigating the effect of *C. sinensis* on tumor development *in vivo* and examine proinflammatory and anti-inflammatory cytokines and markers for macrophage subtypes.

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CONFLICT OF INTEREST
None

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